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SECTION V
BIOLOGICAL SCIENCES

THIRD SERIES—VOLUME XLIX—SECTION V

JUNE, 1955

OTTAWA
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SECTION FIVE

PRESIDENTIAL ADDRESS

The Balance of Bacterial Virulence

E. G. D. MURRAY, F.R.S.C.

BACTERIAL virulence is an excellent example of a biological term of seductive simplicity with remarkable simulation of experimental verification. It trips lightly off the tongue, and, like a constant in the mathematical treatment of statistics, it brings derived configurations into seeming agreement to explain difficult situations. Thus the outbreak of an epidemic would be due to the introduction of a virulent strain of the causative organism and the differences in severity of cases of the same disease would be determined by degrees of virulence of the bacterial cause. With the isolation and maintenance of pure cultures of bacteria in the laboratory and their proven association with particular kinds of disease, it became an easy transition to endow the bacteria themselves with this hypothetical property of virulence. It was an engaging assumption because it ascribed to one source an integrating function which satisfactorily accounted for a variety of discordant observations in the epidemiology, incidence and course of infections.

Advances in the prevention or cure of infectious diseases of man, domestic animals and plants make spectacular news of world-wide interest, because these diseases force themselves on our attention by their ever present threat which frequently culminates in dire realization. A tremendous contribution to our knowledge of the processes of bacterial infection has been derived from the production of disease in experimental animals, using pure cultures of pathogenic bacteria. Although the form of the experimental disease is frequently not identical with that occurring naturally in man, it none the less has character and quality to provide information not otherwise obtainable.

Experiments of this nature have shown, among other things, that different cultures of the same species of microbe are not equally infective to a selected kind of animal under identical conditions. This effect was soon measured in terms of the smallest number of bacterial cells, or weight or volume of culture, that would cause a fatal infection, and this became known as the Minimal Lethal Dose (M.L.D.). It became evident that in making this measurement, account had to be taken of the age of the culture, the kind of medium and the conditions under which it was grown. The effect is also influenced by the species of animal used and the route by which it is infected (by mouth, subcutaneously, intramuscularly, intravenously, intraperitoneally, intracerebrally, intracutaneously, and so forth); often the relation of

dose to weight of animal is important. The M.L.D. is rather more difficult to determine than that which will kill 50 per cent of animals (LD 50), which then becomes subject to analysis to decide whether the result is "statistically significant." But, with all these and other complications, the lethal dose was largely accepted as a measurement of *virulence* and was regarded as expressing a vital peculiarity essentially a property of the bacterium itself.

So simple a process of thought and of investigation easily explored the ever widening range of pathogenic bacteria and tested the variations shown by innumerable isolations of any selected species, to accumulate a mass of valuable information which it was not always easy to interpret. Expressed in terms of this concept, it cannot be denied that certain genera, even certain families of bacteria, are consistently more virulent than others. The organisms of the tribe to which the cause of bubonic plague belongs are all pathogenic to one or another warm-blooded animal, and some of their most active members are so infective that the introduction of one single cell into a susceptible host will cause fatal disease. But such a feature is not necessarily a character common to all species of a genus; for example, a single *Mycobacterium tuberculosis* will cause fatal tuberculosis in a susceptible host, but no known laboratory animal is the worse for the injection of vast numbers of leprosy bacilli from human cases, and other members of this genus cause disease only in hosts of particular species, while yet others have not been found to cause any disease at all. In the genus *Bacillus* only one of over thirty species causes disease in mammals, and that is the redoubtable anthrax, but, though others of this genus are fatal to insects, most of the species are no more than a nuisance to industry or perhaps helpful to various human interests.

As mentioned regarding leprosy, there can be very stringent host restrictions, so that there are many micro-organisms that are peculiar to man, or cattle, or rodents, or some other particular host. This is a situation familiar to zoologists in their study of animal parasites and best illustrated by those with different stages of their life history in different hosts. But it is seen in bacterial diseases too, and most particularly in some of those transmitted by blood-sucking arthropod vectors: the spirochaetes of relapsing fevers are a good example. This is essentially part of the problem of susceptibility and resistance, but it comes into the question of virulence not merely to enable us to select suitable experimental animals, but particularly in a way involving host-parasite relations.

It is interesting to contrast the characteristic pathogenicity of two species of the genus *Neisseria*, both obligatory and important parasites restricted to man and sufficiently like one another that they are difficult to tell apart when found in certain parts of the body. The gonococcus invariably causes disease whenever and wheresoever it is present, and, though there is some variation in severity, with a tendency to chronicity, it finds no natural resistance to infection and no spontaneous cure. The meningococcus by contrast is often present abundantly in its preferred site without causing any

noticeable ill effect, it disappears completely and rapidly from the majority of people it infects, and it is only in a small percentage of people that it either causes severe illness or persists harmlessly for prolonged periods. Both kill experimental animals with overwhelming doses by special routes but without differentiating character and it is difficult to assign to either a measurement of virulence in terms of a M.L.D.

A measurement of virulence is often as impossible to make in terms of the lethal dose either when the animals used are highly susceptible and infection with very few (1 to 5 or so) organisms is fatal or when the animals are highly resistant and doses have to be excessively large. It is also impossible to determine virulence in this way, when disease is of slow development and a few organisms cause the same degree and type of disase as do thousands of millions, as is the case with the tubercle bacillus and *Staphylococcus pyogenes*. Nevertheless, the natural disease may evidently indicate variations and features such as it is sought to measure. The frightening infectivity and fatality of cholera, which is a peculiarly human disease, is disappointingly futile in experiments with laboratory animals.

Using bacteria which lend themselves to reasonably reliable measurement of their lethal dose, with the co-operation of convenient laboratory animals, it is learned that different strains of the same species often vary greatly one from another in virulence. Though this might well be expected, it is surprising that this measured virulence does not invariably coincide with the degree of severity of the disease in the patient from whom the strain has been isolated. Although strains of low virulence may be isolated from fatal cases and strains of high virulence from mild cases, there is an additional indication of quality that the M.L.D. in experimental animals does not reveal. When any cultures of meningococcus (*Neisseria meningitidis*) are inoculated into the clotting blood of the majority of human individuals the organisms are killed within twenty-four hours at 37°C. However, the blood of a few people will not kill cultures freshly isolated from cases of meningitis but will kill cultures well established as laboratory strains. There is a small number of people, not more than 2 per cent, whose blood will not kill even the longest established tame laboratory cultures in this way.

We find these evident differences between individuals of the host species in natural disease (such as cerebro-spinal fever and poliomyelitis) as well as under experimental conditions. Races of laboratory animals are bred which are more resistant or more susceptible to infection and this can be to particular diseases. It is probable that susceptibility and resistance become characters of natural selection in the course of evolution, for it is well known that Algerian sheep are strangely resistant to anthrax and there is a race of cattle in central Africa unduly susceptible to rinderpest. There are indications of selection by disease to produce human communities with greater tolerance, as is illustrated by tuberculosis and syphilis and some others.

When bacteria are grown in the laboratory with successive subculture on the ordinary run of laboratory media, many kinds become markedly lowered

in virulence as measured by the M.L.D. Sometimes this effect is produced quickly, so that animals can only be infected by very recently isolated strains, and sometimes it is a reasonably slow operation. Kinds of bacteria which produce spores will often retain virulence in their spores which is manifested by the germinated cells. But cultures can be changed purposively, as Pasteur did by growing anthrax at 40°C. to produce his attenuated strains for his first vaccine to protect the sheep of France.

Old stock strains of most bacteria have lost their virulence with prolonged culture and the effect may be brought about differently in different species. It depends to some extent on the way the kind of bacterium produces the disease. Those which depend on the production of a toxin may or may not lose this ability, which is almost a personal attribute, just as some men are good athletes while others may be good scholars as well. Those that depend largely on a well-developed capsule protecting them from phagocytosis by the animal's leucocytes and reticulo-endothelial cells may not in culture make effective capsules and may easily lose the knack. Apart from the immunological importance of the capsular polysaccharides, which is an ancillary complication in the study of virulence, there is much definite proof of well-developed capsules affording bacteria protection from phagocytosis. Variants of bacteria can be produced that are devoid of their natural capsules and these are then avirulent and easily engulphed by leucocytes; but they can then be made to restore their capsules and so become virulent again. The temporary removal of the capsule by specific enzymes demonstrates beyond doubt its importance in resistance to phagocytosis, but quite well capsulated forms may be avirulent and mere multiplication of bacteria in the body does not explain the lesions or the fatality of the disease.

There are beyond doubt intrinsic bacterial attributes that are essential to their producing disease and these are significant to this aspect of the process that is measured by the M.L.D. At best, the conditions we provide in test-tubes for bacterial growth are probably not much more than just tolerable to those that are most confined to pathogenic parasitism and therefore most fastidious. It is to be expected that with their great rate of multiplication they survive by the selection of the most adaptable cells, that is, in the test-tube, those most saprophytic of their kind. Otherwise, too, for the synthesis of their greatest needs for pathogenicity we may not supply their requirements in our media, and this possibility is very often overlooked. It has been shown with the meningococcus that by the proper adjustment of ingredients a medium is easily made to give cultures of many times greater virulence than cultures of the same strain on other media. The most important ingredient is contained in a tryptic digest of meat and the required quantity has to be titrated by the M.L.D. of cultures grown on varied concentrations, but once that is done for a batch it remains unchanged. On ordinary media the meningococcus dies out in a few hours with loss of virulence on subculture, but on the specially adjusted medium it survives many months, and, most importantly, the virulence in terms of M.L.D. for mice

remains high and unchanged for at least two and a half years. A similar though less worked out effect has been shown with other bacteria. There is thus a nutritional dependence to virulence of great importance though it is not yet understood, and this has attracted very little attention from either bacteriologists or biochemists.

Interesting and suggestive as these examples of *in vitro* results may be, the final test rests with the more natural circumstances provided by the living animal. There is no doubt that strains of differing degrees of virulence are isolated from natural disease independently of the severity of the case. This does not preclude a stability of virulence-contributors which are directly a property of the strain of bacterium and not variable under natural conditions, even though diminishable by the deprivations of our culture methods.

It has been established from early days that bacteria of low virulence can be quickly raised to a high order by passing them through animals in series. The success of the operation varies with the bacterial species; some, like the pneumococcus, respond extravagantly and others, like the tubercle bacillus, reluctantly if at all. A pneumococcus culture in broth having a M.L.D. of, say, 0.5 cc. can by serial passage in mice be raised in virulence to have a M.L.D. of one ten-millionth of a cc., tested by intraperitoneal inoculation in mice, which far transcends the effect of any known culture method.

Even with an easily transformed organism like the pneumococcus, animal passage may fail when hap-and-chance dosage is used, but it succeeds with the same culture strain when the dose in each succeeding stage in the series is carefully adjusted to the ever-diminishing amount constituting one or two M.L.D.'s. By the same method of animal passage but using a series of overwhelmingly large doses of a highly virulent strain, an animal at the end of the series now yields a culture of low virulence. In this procedure the animal is, as it were, turned into a mere culture medium by overwhelming its natural defences against infection, so that there is no competitive selection of the bacterial cells which are allowed to reproduce. In this way there is a survival of the unfit, from the parasite's point of view, and these, like feeble-minded humans, may breed most readily.

It might be urged that the pneumococcus does not naturally infect mice and that, though it is an improvement on culture, this experiment may be artificial. But *Listeria monocytogenes* isolated from natural disease in rabbits was quite easily both raised to high virulence and depressed to low virulence by passage in rabbits. There is, then, a quality proper to bacteria that can be augmented or diminished in the activity expressed by this measurement of virulence and that is independent of the susceptibility or resistance of the host.

Not all kinds of bacteria respond in this way to such treatments. There are those whose injurious or fatal effects depend on their production of a toxin and their powers to do so are not altered by animal passage, and there is probably no justification to speak of them as virulent, however fatal

they may be. Such are the causes of diphtheria, lockjaw, and botulism, for example. Only it must be emphasized that this black and white division is not strictly applicable for there are many shades of grey represented; these may be exemplified by some of the gas-gangrene bacilli and the staphylococcus, which present both possibilities. Also, members of the typhoid-coli family seem to depend largely upon their structural antigenic components, which are altered in an entirely unrelated way and are not materially changed by passage. Their virulence is probably more a question of qualities of which studies of bacterial heredity are proving most revealing, but, even so, experiences reported with vaccines made of recent years indicate the loss of some important property with attempted purification of antigens.

Attenuation by animal passage is not a new procedure and the following are some of the most noteworthy examples: Pacteur induced a progressive attenuation of rabies by establishing a fixed virus in the central nervous system of rabbits, which was then subjected to degrees of desiccation for use as his rabies vaccine. By this means each successive dose of living vaccine given to people bitten by rabid dogs was progressively more virulent for the rabbit than the dose preceding. Vaccinia vaccine is quite probably a smallpox virus attenuated for man by adaptation to cattle. Hence it causes a localized, mild but immunizing infection with spontaneous cure. An adaptable strain of rinderpest virus was successfully established in the embryonated hen's egg and after a number of passages it became fixedly reduced to harmless infectivity for cattle. As such, it proved to be a very effective immunizing antigen to protect cattle against rinderpest. This manner of attenuation of virulence for the natural host by passage through an unrelated species, for which it acquires a marked infectivity, is not confined to viruses; it occurs too with bacteria, such as the passage of swine erysipelas (*Erysipelothrix rhusiopathiae*) to increase its infectivity for rabbits, which reduces its virulence for swine.

These empirical observations are of very special interest because they concern modification of virulence in a way involving essential provisions contributed by both parasite and host, each of which seems to be materially affected. Thus either the case is very different to those previously considered, or else in those others the host contribution is masked by the exaggeration of the influence of the parasite. Be that as it may, these special cases explore the question of susceptibility and resistance, which is perhaps the most important problem posed by infectious disease and at the same time the one about which we know least.

Attenuated living bacterial vaccines have been used for the protection of man and animals against infections to which they are naturally subject. Pasteur used an attenuated anthrax vaccine for sheep, and since then attenuated bacteria have been used to immunize cattle against brucellosis, and to protect man against bubonic plague. The most extensively employed of all is smallpox vaccine, and after that B.C.G. as at present used to protect man against tuberculosis. This may not be quite as simple in principle

as it might appear to be at first sight. When specific types of pneumococcus, which possessed identifiable host selective virulence-characters, were turned to the "R phase" by the loss of their capsule they were no longer virulent. But when these very same derived "R" cultures were induced to form immunologically specific capsules of another than their original type and so revert to virulence, the host selectiveness of this new virulence was that of the strain from which the "R" phase was derived and not that of the strain providing the capsule. There appears therefore to be a cellular factor which has some determining influence on virulence. Although this cannot be exhibited directly all by itself and needs the coexistence of some activator, it certainly persists intact through the process of attenuation of virulence.

There are kinds of bacteria whose habit and behaviour lead to the expectation of an easy measurement of their M.L.D. and an easy raising of their virulence by animal passage, but experiment proves that neither one can be realized. For many years it was conceded by the greatest authorities that the meningococcus presented exactly these difficulties. The suitable adjustment of the culture medium overcame the first obstacle, but, although the M.L.D. of a culture of meningococcus could then be measured reliably, its virulence could not be raised consistently by animal passage. As if by accident, a single passage would sometimes yield a culture of increased virulence. With this lead and the supporting observation that the more virulent the strain of meningococcus the more resistant it was to phagocytosis and the better it could survive ingestion by phagocytes, bacteriolytic cell-free extracts of polymorphonuclear leucocytes were made. By a suitable adjustment of the ratio of this active extract of leucocytes to the number of bacterial cells and by the proper use of time, cultures treated *in vitro* yielded strains of markedly increased virulence with desirable regularity. This was not a mere survival of the occasional bacterial cell capable of resisting the action of the leucocyte extract, for the effect was strictly progressive with time and the use of too great a proportion of extract failed to be effective.

Here then is a selective host element against which the meningococcus must normally strive for its existence, and then those bacterial cells that can most effectively resist will reproduce; but of these, those which have the propensity to breed cells of greater efficiency will produce a strain of increased virulence. There are those who work with antibiotics and who deny even such a possibility, preferring a preconception of sporadic individual mutants fully endowed with unchanging resistance. They are, as it were, "Fed on extravagant poetry, and lit by such a dazzle of old fabulous tales that common things are lost and all that's strange is true because 'twere pity if it were not"! Why fly to this extremity of obstinate belief, because, forsooth, the situation concerns bacteria, when in three generations mice can be bred to resist natural infections or even mineral poisons, and wheat can be bred resistant to rust.

The availability of virulent cultures of meningococcus and a dependable titration of their M.L.D. allowed of experiments to test the importance of

phagocytosis and purposely to vary its effectiveness to protect against infection with definite relation to the measure of virulence of the culture. A selected large intraperitoneal dose of low-virulence organisms failed to kill, although it caused peritonitis, and all the bacteria were found contained in healthy-looking leucocytes. But a comparable dose mixed with a sterile suspension of carmine was fatal and this result was accounted for by nearly all the bacteria being free outside the leucocytes which were crammed with carmine particles. The chief defence of the animal under these circumstances was blocked by the leucocytes' selective preference for carmine leaving the bacteria free; and in fact a very much smaller dose than usual of the culture was fatal in the presence of carmine. When animals were prepared, by a previous intraperitoneal injection of aleurone, to accumulate large numbers of healthy, active leucocytes, it took multiples of the normal M.L.D. of a highly virulent strain to kill. This was more difficult because of the tendency of the virulent strain to resist phagocytosis. Accumulation of leucocytes by other means, such as injecting the salivary glands of larvae of *Gastrophilus equi*, which collected even larger numbers of leucocytes than did aleurone, but deprived them of phagocytic activity, did not afford protection against cultures of either high or moderate virulence.

By using measured minimal lethal doses in these experiments, the *apparent* virulence of a culture could be shifted up or down by very simply interfering with the defence processes of the animal, without in any way altering the *actual* virulence dependent on the bacterium itself. These experiments are significant because the polymorphonuclear leucocytes responsible for protection were abundantly present in every instance. In this they differ from the practice of rendering animals susceptible to certain infections by suppression of leucocyte production with benzene or X-rays or with the complexities of treatment with cortisone.

There is no present agreement among bacteriologists on a definition of *virulence* and the literature abounds with terms used evasively, such as pathogenicity and invasiveness, which by lack of definition cause confusion. The introduction of toxigenicity into the complexity is unnecessary and merely serves to make the scope more diffuse. Toxin production is a physiological activity of certain kinds of micro-organisms contributing to their pathogenicity but it is an inherent attribute and essentially it varies quantitatively only with the strain, the availability of certain requirements, and, in one instance, infection with particular lysogenic bacteriophages. In describing the diphtheria bacillus, even the most insistent authors use the terms toxin production and virulence as synonymous, but, since virulence is less precise and since many other factors associated with it do not apply, its use is without meaning or purpose in this instance. It would be impossible to ascribe virulence to *Clostridium botulinum* and difficult to do so for the tetanus bacillus but quite evidently in gas-gangrene *Clostridium perfringens* may well exhibit both virulent invasiveness and toxigenicity.

Pathogenicity is an all-embracing term and expresses no more than a potentiality to cause disease by some means in an animal, plant, or bac-

terium. An organism need not be a parasite to be pathogenic and it need not be virulent or invasive, for all these are excluded in the case of *Clostridium botulinum*, which produces the most poisonous toxin known. Many parasites are not pathogenic though they can only live in or about the body of a host; some are even beneficial. Pathogenicity may be contributed to by virulence and invasiveness and, either with these or independently of them, be determined by toxigenicity. The term pathogenicity bears no relation to taxonomy or classification but it expresses succinctly an observed behaviour of a recognized organism and means no more than does the statement that some mushrooms are poisonous.

If we confine the meaning of invasiveness to express the habit of certain bacteria to spread in the tissues, or to distribute themselves throughout the body, it has a useful descriptive application. It could then be said that the typhoid bacillus, certain streptococci, the gonococcus, the tubercle bacillus, the syphilis spirochaete, and others are invasive, and contrarywise it is quite definite that the cholera vibrio, the diphtheria bacillus, the dysentery bacilli, and the tetanus bacillus are not invasive. It is quite evidently a capability some bacteria have and others have not. This is quite irrespective of taxonomy and classification, for even in one genus like Salmonella some species are and others are not invasive. Invasiveness most certainly contributes to virulence but the two terms are not coextensive in meaning if organisms like the cholera vibrio are conceded to be virulent. In any case the two need not be confused and invasiveness has a definite use and meaning, distinguishing clearly between behaviours of important pathogenic bacteria.

Despite many pathologists and bacteriologists using the terms pathogenicity, invasiveness, toxigenicity, and virulence as synonymous, there is clear good reason to distinguish between them without any confusion. Some authors think these terms "deserve to be dropped" but retain pathogenicity. Miles regards virulence as a particularized case of pathogenicity and as such makes a distinction that is rendered artificial by recognizing the complex relations between parasite and host, and at the same time saying "but if it is to be any use to us in the analysis of microbial pathogenicity, we must invent some way of ignoring the host if we are to avoid fruitless circular arguments." It seems to me such an achievement would be as rewarding as playing a piano in a vacuum.

The only one of the terms in question that I find difficult to define clearly is virulence. If any contributory influence of either pathogen or host is ignored, the possibility of understanding the intricately balanced system of antagonistic and synergistic processes becomes hopelessly difficult. These processes vary with each different assortment of pathogen and host and may be distorted by the inadequacy of our techniques for both their recognition and their measurement. Because the pattern in any selected system cannot yet be satisfactorily analysed, the end-result of a defined mortality rate has to be accepted as a measure of virulence. This amounts to a drastic finality in using the 100 per cent M.L.D. which risks incongruous weighting of the pathogenicity, or else a hesitant uncertainty in using the 50 per cent

L.D. because it is unknown why one half of the animals die and the other half contrive to survive. Dependence on mortality is not satisfactory for organisms which cause localized, mild, or chronic disease, such as angular conjunctivitis, acne, soft chancre, or some forms of diarrhoea; and for that matter more serious pathogens such as those of syphilis, cholera, and gonorrhoea.

The existing confusion is more than a matter of semantics and the first requirement is to know whether there is any foundation for a concept of virulence that is not already covered by another more easily defined term. Pathogenicity is too wide and includes invasiveness, communicability, toxicity, and something more than the sum of these. Bacteria are able to take advantage of all and sundry circumstances to secure their distribution, and pathogenicity at times involves something other than invasiveness and toxicity, as is indicated by regionally selective localized disease (angular conjunctivitis, acne, pharyngitis, and perhaps furunculosis). But vastly more impelling is the need for a concept that takes into account the diversity of competing influences contributed simultaneously by both parasite and host. For it is abundantly clear that the balance of these determines the character and the severity of infections and that either parasite or host may contribute to its own destruction.

Even though every element from whatever source cannot be identified, *virulence* can be defined as the *resultant* of two opposing systems of forces; those of the pathogenic parasite striving to secure its own survival set against all the odds the host can muster to eliminate the cause of its disease. No contributing influence developed by either system can be ignored, for the host is not merely a culture medium and the living bacterium is not at any time an inert particle. Where a principal component of either set of forces can be modified, the tendency of the resultant will change to favour either the parasite or the host, as has been illustrated by the experiments with the meningococcus. Dependence on fatality not only limits the sensitiveness of the estimation of virulence but restricts adequate testing of many kinds of pathogenic bacteria, even of a number which cause serious disease.

The advantage the flexibility of this definition imposes is the use of whatever criterion most suits the parasite or the form of the disease in any selected host. Whether the observed end-point of a test be death or merely diarrhoea, time of survival of the parasite or provocation of a cellular response, the use of the term virulence in this sense remains valid and significant. Most of all, this recognition of a balance allows of the integration of any number of varied influences, and the discovery of new effects does not in any way detract from old observations.

So, we who must read as we run may secure by this device that as the moving finger writes we do not get a blurred impression, and, above all, it provides that the record we make of our observations will be clarified and expanded by whatever new knowledge we may gain.

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SECTION FIVE

Studies in *Potentillae* of High Latitudes and Altitudes

PIERRE DANSEREAU, F.R.S.C.

I. Introduction[1]

THE genus *Potentilla* L. of the Rosaceae belongs to the subfamily Rosoideae, the tribe Potentilleae and the subtribe Potentillinae. The latter, according to Focke (1894) also contains: *Fragaria, Horkelia, Ivesia, Sibbaldia, Potaninia, Chamaerhodos, Horkeliella, Purpusia, Duchesnea.* The last-named is included by Wolf (1908) in *Potentilla*, of which he describes 305 species. A dozen or more have been added since that time but no general revision of the genus has been attempted.

The Basic Units

Wolf (1908) recognizes 31 fundamental units, which he calls greges. These, in turn are assembled into 6 subsections (some of which are divided into series), and the latter in 2 sections. Some of the greges have been, or are considered by other authors to be, distinct genera (see synonomy in Wolf 1908):

Fruticosae	= *Pentaphylloides* Duh.
Xylorrhizae	= *Trichothalamus* Lehm.
Palustres	= *Comarum* L.
Tridentatae	= *Sibbaldiopsis* Rydb.
Rupestres	= *Drymocallis* Rydb.
Tormentillae	= *Tormentilla* L. p.p.
	= *Duchesnea* Sm. p.p.
Anserinae	= *Argentina* Lam. p.p.

By modern writers the genera *Pentaphylloides* Duh. (= Dasiphora Raf., see Löve 1954), *Comarum* and *Duchesnea* and even *Argentina* are often maintained, whereas the others seem to be recognized only by "splitters." It is worth pointing out that the latter tendency is no longer frowned upon as severely as it once was. It turns out that such sharp observers as Rydberg and Small (or even Rafinesque) have drawn attention to units that have a very real genetic and ecological existence and that the contemporary experimental taxonomists are able to confirm the validity of many of their taxa (see Clausen 1951). If the "comparium" be substituted for the genus, *Potentilla* contains several of these. All the genera named above will probably turn out to be genetically distinct. It also stands to reason that the half-

[1]Contribution no. 1004 from the Department of Botany of the University of Michigan.

century that has elapsed since Wolf's system was proposed (1908) has revealed unsuspected or undetected affinities, and that some greges have had to be reshuffled to some extent. In the grex *Graciles*, for instance, Clausen, Keck, and Hiesey (1940) include *P. diversifolia* (of the *Multijugae* sensu Wolf), whereas they transfer *P. breweri* (erstwhile of the *Graciles*) to close proximity with *drummondii* (of the *Multijugae*), since they consider the two, on experimental evidence, to form a single cenospecies. Other reassemblages will certainly follow upon further investigation.

Chromosome Numbers

Table I lists the known diploid chromosome counts for species, subspecies, or varieties of *Potentilla*, arranged and numbered according to Wolf's system. The names of the authors and year of publication of the cytological work are given, whereas the full reference is cited in the bibliography.[2]

Table II shows a re-arrangement of these data by greges, omitting species enumeration and citations. There is no doubt that 7 is the basic (or x) number (as it is in several other Rosaceous genera: *Duchesnea, Fragaria, Sibbaldia* of the same subtribe; *Geum, Agrimonia, Sanguisorba* of the same tribe; *Rosa, Rubus* of the same subfamily). Ploid levels 2, 4, 5, 6, 7 all show only multiples of that number (with the single exception of a reported *P. tormentilla* with 32 chromosomes). At higher levels there is quite frequent aneuploidy although some perfect 8-, 9-, 10-, 11-, 12-, 15-, and 16-ploid numbers occur.

Counts are available for 92 taxa representing at least one species in each of 27 of the 31 greges. In 10 greges, the low diploid number 14 is found. In the *Speciosae, Nitidae, Crassinerviae, Rupestres*, and *Fragarioides*, that is the only known number. In the *Fruticosae, Multifidae, Argenteae, Rivales, Aureae*, higher levels have also been reached. This even occurs frequently within a single species,[3] as in *fruticosa* (*Fruticosae*), *nivea* (*Niveae*), and *argentea* (*Argenteae*). Most greges are strictly polyploid, as far as known. The *Graciles, Hematochroae, Niveae, Chrysanthae*, and *Multijugae* are all at least hexaploid.

No triploids ($3x = 21$) have ever been recorded. Tetraploids ($4x = 28$ in 30 taxa) and hexaploids ($6x = 42$ in 37 taxa) show the highest incidence, and no aneuploidy at all. This latter phenomenon first appears at

[2]It is hoped that this repertory is reasonably complete. Unfortunately, since the discontinuation of Gaiser's and of Tischler's catalogues, no single compendium is available. I shall be most grateful to those who will kindly draw my attention to any errors in this list and who will add new counts to it.

[3]Although a different chromosome number may not in itself provide sufficient evidence for declaring specific status, such a procedure has been in part adopted in the following cases: *P. fruticosa* (2n = 14, 28), *P. arbuscula* (2n = 14); *P. floribunda* (2n = 14); *P. nivea* (2n = 56, 63, 70), *P. chamissonis* (2n = 77); *P. norvegica* (2n = 70), *P. monspeliensis* (2n = 56). In all of these cases the morphological and geographical differences, already considered significant by taxonomists, were merely confirmed by cytology (see Löve, 1954).

TABLE I

Diploid Chromosome Numbers in Potentilla

The names and numbers of greges and species are those listed in Wolf (1908), although in some cases (viz. *P. emarginata*) another name (*P. hyparctica*) has been demonstrated to be the valid one. Where the name under which the number is cited by the author of the chromosome count is different from Wolf's, mention is made in parentheses. This sometimes indicates a mere difference in nomenclature and sometimes refers to a taxonomic split. Names in *italics* refer to taxa not known to Wolf or not recognized by him. The abbreviations for the authors' names are as follows: BL, Böcher and Larsen; CKH, Clausen, Keck, and Hiesey; DJA, Darlington and Janaki Ammal; E, Erlandsson; F, Flovik; LL, Löve and Löve; M, Müntzing; MM, Müntzing and Müntzing; P, Popoff; Sh, Shimotomai; T, Tischler. Complete references will be found in the bibliography, thus: LL48 = Löve, A. and D. Löve, 1948, etc. All numbers cited here do not necessarily indicate an original determination.

GREX		SPECIES		CHROMOSOMES	
No.	Name	No.	Name	2n	Authority
1	Fruticosae	1	fruticosa L.	14	Sh 30
				28	LL 48
		1A	*arbuscula* D. Don	14	Sh 30
		1B	*floribunda* Pursh	14	L 54
2	Bifurcae	3	bifurca L.	56	P 35
5	Palustres	9	palustris Scop.	28, 42, 64	LL 48
6	Tridentatae	10	tridentata Scop.	28	Sh 30
8	Speciosae	17	speciosa Willd.	14	Sh 30
9	Nitidae	22	nitida L.	14	T 50
		23	alchimilloides Lap.	14	Sh 30
11	Crassinerviae	28	valderia L.	14	Sh 30
13	Fragariastrae	37	alba L.	28	P 35
		38	montana Brot.	28	P 35
		39	sterilis Garke	28	LL 48
14	Rupestres	41	rupestris L.	14	CKH 40
				14	P 35
				14	Sh 30
		42	arguta Pursh	14	P 35
				14	CKH 40
		43	glandulosa Lindl. (s.l.)	14	Sh 30
				14	CKH 40
		47	geoides MB.	14	P 35
		48	calycina Boiss. & Bal.	14	Sh 30
15	Multifidae	52	pulchella R. Br.	28	LL 48
		54	multifida L.	28	LL 48
				28	P 35
				42	Sh 30
		71	bipinnatifida Dougl.	42	Sh 30
		73	chinensis Ser.	14	Sh 29
		74	niponica Th. W.	28	Sh 29
		75	pensylvanica L.	28	Sh 30
16	Graciles	79	hippiana Lehm.	42	Sh 30
		84	breweri Wats.	72–73, 99	CKH 40
				100, 102	CKH 40
		89	crinita Gray	84	Sh 30

TABLE I (*Continued*)

GREX		SPECIES		CHROMOSOMES		
No.	Name	No.	Name	2n	Authority	
		92	pulcherrima Lehm.	70, 71, 108	CKH	40
		93	gracilis Dougl. ssp. *nuttallii* (Lehm.) Keck	70	Sh	30
				84	P	35
				52, 54, 54–55	CKH	40
				56, 57, 58, 59	CKH	40
				62, 63, 64, 68	CKH	40
				70, 72, 66–74	CKH	40
				78–80, 84–87	CKH	40
				84, 101, 103, 109	CKH	40
		95	pectinisecta Rydb.	42	CKH	40
		96	flabelliformis Lehm.	60–65, 62, 64, 66	CKH	40
				70	Sh	30
17	Haematochroae	105	nepalensis Hook.	42	Sh	30
		108	haematrochrus Lehm.	112	Sh	30
		111	argyrophylla Wall.	56	Sh	30
				56, 63	P	35
		112	atrisanguinea Lodd.	56	Sh	30
				56, 63	P	35
		112a	sibthorpiana	84	Sh	30
18	Niveae	113	nivea L.	70	Sh	30
				63	BL	50
				56	LL	48
		113A	*chamissonis* Hult.	77	LL	48
				56	BL	50
		115	villosa Pall.	42, 49	M	31
19	Argenteae	125	dealbata Bge.	42	Sh	30
		126	argentea L.	42	Sh	30
				42	P	35
				14, 28, 35, 42	MM	44
				14, 28, 35, 42, 56	LL	48
		126	argentea L. var. incanescens (Op.) Focke	35, 42	MM	44
		127	meyeri Boiss.	42	Sh	30
		128	canescens Bess. (& vars.)	42	Sh	30
19a	Collinae	136	sordida (Fries) Zimm.	42	Sh	30
		139	sommieri Siegfr. & Kell.	42	Sh	30
		142	thyrsiflora (Hüls.) Zimm. (sub collina)	42	Sh	30
				42, 84	MM	43
				42, 70, 84	M	31
				35, 42, 84	T	50
20	Tanacetifoliae	159	pimpinelloides L.	70	Sh	35
		160	visianii Panc.	28	P	35
21	Rectae	165	recta L. (& vars.)	42	Sh	30
				28	P	35
		166	laciniosa W. K.	28	Sh	30
		167	transcaspia Th. W.	42	Sh	30

TABLE I (*Continued*)

GREX		SPECIES		CHROMOSOMES		
No.	Name	No.	Name	2n	Authority	
		169	hirta L.	28	Sh	30
				28	P	35
		173a	taurica Willd. var. nicicii (Adam.) Th. W.	42	Sh	30
22	Rivales	175	supina L.	28	Sh	30
		178	dombeyi Nestl.	42	Sh	30
		182	norvegica L. var. genuina Th. W.	70	LL	48
		182A	*monspeliensis* L.	56	L	54
		187	intermedia L.	28	Sh	30
				28	Sh	35
		188	wallichiana Del. (sub kleiniana)	14	Sh	29
23	Persicae	200	kurdica Boiss. & Hoh.	56	P	35
		201	nevadensis Boiss.	28	Sh	30
				28	Sh	35
24	Grandiflorae	214	grandiflora L.	28	T	50
		216	buccoana Clem.	28	Sh	30
		217	pyrenaica Ram.	28	Sh	30
		219	umbrosa Stev.	70	Sh	30
25	Chrysanthae	220	chrysantha Trev.	42	Sh	30
			(& var. normalis)	42	Sh	35
		221	thuringiaca Bernh.	42	Sh	30
		221a	thuringiaca Bernh. var. elongata Th. Wolf.	42	Sh	30
			(sub parviflora ssp. goldbachii)	42	LL	48
26	Muitijugae	235	drummondii Lehm. (ssp. *typica*)	92, 96, 100, 104	CKH	40
				105, 106–108	CKH	40
		235a	drummondii Lehm. (ssp. *bruceae* (Rydb.) Keck)	64, 69, 70	CKH	40
				62–73, 71, 79, 87	CKH	40
		244	diversifolia Lehm.	82–84, 90–91, 101	CKH	40
27	Ranunculoides	249	matsumurae Th. W.	28	Sh	29
28	Aureae	261	dubia (Crtz.) Zimm.	14	T	50
		266	emarginata Pursh	42	F	40
			(sub *hyparctica*)	42	LL	48
		267	gelida C. A. Mey.	42	Sh	30
				42	Sh	35
		272	alpestris Hall.	28 42	DJA	45
				42	Sh	35
		272a	alpestris Hall. var. levieri Th. W.	42	Sh	35
		272A	*crantzii* Beck	42(49)	LL	48
		273	aurea L.	14	Sh	35
				14, 28, 56	T	50
		275	opaca L. (sub heptaphylla L.)	14	MM	44
				14	LL	48
		282	verna L. (p.p.) et auct. plur. (sub tabernaemontani Asch.)	42, 84	M	31
				42, 49	MM	42
				28, 42, 49, 63	T	50

TABLE I (*Continued*)

GREX		SPECIES		CHROMOSOMES		
No.	Name	No.	Name	2n	Authority	
		284	arenaria Borkh.	35, 42	LL	48
				56	LL	48
		287	velutina Lehm.	42	Sh	30
29	Fragarioides	289	fragarioides L.	14	Sh	29
		290	freyniana Bornm.	14	Sh	30
30	Tormentillae	291	tormentilla Neck.	28	P	35
			(sub erecta)	28, 32	LL	48
		292	procumbens Sibth.	42	T	50
		293	reptans L.	28	Sh	30
				28	LL	48
31	Anserinae	299	anserina L.	28	P	35
				28, 42	LL	48
		299A	*egedii* Rydb.	28, 42	E	42
		299Aa	*egedii* Rydb. var. *groenlandica* (Tratt.) Pol.	28, 35, 42	E	42

the 5-ploid level (only one case) and becomes quite common at the 8-ploid level and upwards. No less than 9 greges range through 7 or more ploid levels, although some do so discontinuously; three of them span 11 levels.

Geography versus Ecology

The cinquefoils are predominantly boreal, although they are common farther south. The species of tropical latitudes are apparently confined to the highlands, however, where they cross the Equator: *parvula, microphylla, leuconota, papuana* in Borneo and New Guinea; *anserina* and its relatives (the grex *Anserinae* of Wolf, 1908) in Chile, New Zealand, Australia (Victoria), Tasmania, Borneo, New Guinea; *dombeyi* from Mexico to Chile in a discontinuous range at high altitudes. In any strict sense of the word, there are no tropical *Potentillae*. They are very widespread in the warm-temperate to cold forest areas of the Northern Hemisphere, but are distinctly fewer or even locally absent from the prairie, steppe, and desert zones.

A comprehensive geographical analysis of the genus *Potentilla* is not available. But a sampling of boreal regional floras reveals that where it is present at all it is generally represented by several species. Moreover, in most areas, members of two or more greges are to be found. Areas harbouring not only a large number of species but above all a large number of greges (eastern Asia, western North America, southern Europe) may be considered primary centres of differentiation.

It is to be noted that the facts of distribution, as they appear in outline or dots on the maps do not provide us in this case with a clue to the habitats occupied by cinquefoils, which largely avoid the forests that are the zonal key-vegetation.

TABLE II

Distribution of Chromosome Counts in Taxa of the Genus Potentilla

The counts are arranged according to their ploid level and in the order of Wolf's taxonomic treatment. The plus-sign indicates occurrence of the exact euploid number in one taxon; otherwise the aneuploid figure is given. Authorities for counts cited in Table I.

Wolf's (1908) classification			2x 14	3x 21	4x 28	5x 35	6x 42	7x 49	8x 56	9x 63	10x 70	11x 77	12x 84	13x 91	14x 98	15x 105	16x 112
Section	Series	Grex															
I. TRICHOCARPAE																	
A. Rhopalostylae		1. Fruticosae	+ +		+												
		2. Bifurcae							+								
B. Neuratostylae	a. Suffruticulosae	3. Xylorrhizae															
		4. Biflorae															
		5. Palustres			+												
		6. Tridentatae			+		+			64							
		7. Eriocarpae															
	b. Herbaceae	8. Speciosae	+														
		9. Nitidae	+ +														
		10. Curvisetae															
		11. Crassinerviae	+														
		12. Caulescentes															
		13. Fragariastra			+ + +												
II. GYMNOCARPAE																	
A. Clerostylae		14. Rupestres	+ + + + +														
B. Conostylae	a. Eriotrichae	15. Multifidae	+		+ + + +		+ +										
		16. Graciles					+ +		52, 54	60–65	68	78	80, 80	90	99, 100	108	109
									55–56	62, 62	66–74		+ +		100, 101		
									+	+	+ + + +		84–87		102, 103		
		17. Hematochroae							57, 58	64, 64	71, 72						
		18. Niveae					+		59	66	72–73						
		19. Argenteae	+		+	+ +	+ + + + +		+ +	+			+				+
		19a. Collinae				+	+ + +		+ + +	+	+	+					
	b. Orthrotrichae	20. Tanacetifoliae			+				+		+		+				
		21. Rectae			+ + +		+ + +				+						
		22. Rivales	+		+ +		+										
		23. Persicae			+				+		+						
		24. Grandiflorae			+ + +		+ + +		+								
		25. Chrysanthae					+ + + +				+						
		26. Multijugae								64	69	79	82–84	90–91	96, 100	104	
											+			92	101	+	
		27. Ranunculoides			+						71					106–108	
											62–73						
C. Gomphostylae		28. Aureae	+ + +		+ + +	+	+ + + + +	+ +	+ +		+		+				
		29. Fragarioides	+ +				+ + +										
		30. Tormentillae			+ +	32	+										
D. Leptostylae		31. Anserinae			+ + +	+	+ +										
Number of taxa			19	0	30	5+1	37	2	11+6	3+8	11+8	1+2	5+4	0+3	0+9	1+3	1+1

Characteristics of Potentilla

The most striking features of the genus *Potentilla* are probably as follows.

1. *Potentilla is morphologically homogeneous.* This is to say that the total amount of variation is relatively narrow and that species differ in the rearrangement of a small number of vegetative and sexual characters. This would be in contrast to such genera as *Echium, Lobelia, Senecio* which can be considered heterogeneous.

2. *Some Potentilla species are polymorphic.* The small range of character amplitude makes for dubious species boundaries in some groups and in some areas. There have been described a large number of hybrids and it seems that their perpetuation is sometimes ensured by *apomixis.* This pattern resembles the *Mentha, Alchemilla, Rubus,* and *Hieracium* situations. Certainly, as Clausen (1954) has pointed out, partial apomixis is one of the strongest agents of a diversified evolution.

3. *Evolution in Potentilla has progressed through polyploidy.* Not only are many greges on a high ploid level, but species within a grex are on different levels, suggesting recent *euploid* development. This may resemble the situation in *Vaccinium* and *Zauschneria* (Stebbins, 1950). It is interesting that *aneuploidy* seems to be present only in the high polyploids. This presumably confers greater importance upon apomixis, a situation parallelled in *Crepis* (Stebbins, 1950). Polyploidy itself can be expected to play a leading role in the emergence of new units in "new" areas such as the Arctic and Subarctic (Löve, 1953).

4. *Potentillae are very common on open dry sites* and correspondingly infrequent in shady and moist habitats. This general ecological confinement manifests itself in practically all the subgroups of the genus and any departure therefrom presents a major step in adaptation (*P. palustris* and its whole grex, sometimes considered a different genus!). Also, minor variations within this general amplitude reveal particular geographic trends, sometimes involving convergence of several greges. Certainly the capacity to expand into new or unusual habitats is altogether as significant an evolutionary move as the elaboration of new morphological structures.

5. *Potentilla is poorly represented in the warmer and drier regions.* The somewhat helio-xerophytic ecology of *Potentilla* is paradoxically integrated into the broader framework of cold to cool but also rather moist climates. This apparent reversal of requirements needs closer examination in order to verify the application of Boyko's (1947) "geo-ecological law" according to which "The specific topographical distribution (micro-distribution) of an ecotypic plant species is a parallel function of its general geographical distribution (macro-distribution) since both are determined by the same ecological amplitudes." Again the extension of taxa in climatically warm or dry areas represents an evolutionary step of great significance, and in this genus, an unusual one (*P. glandulosa* in low rainfall areas of western California, *P. saxosa* in the Colorado desert).

6. *Geographical distribution of Potentilla is very wide.* Some species span a whole continent, and not a few greges cover a substantial part of the genus' range. A study similar to that of Pojarkova (1933) for the maples would reveal the centres of differentiation. These may turn out to be near the focal points for *Acer*, an interesting parallel, inasmuch as the two genera show a good deal of geographic coincidence but almost no ecological overlap? On the whole, the maples are linked to rather stabilized, complex, often climax plant communities, whereas the cinquefoils are almost always members of somewhat transient or at least pioneer communities.

The Problem of Cold-Climate Species

It is not intended, at the moment, to undertake a revision of the whole-genus, but rather to concentrate upon the status and behaviour of those taxa, of quite various phylogenetic origin within *Potentilla*, that have reached the cold areas of the earth. (These areas have all been somewhat recently disturbed climatically.)

Table III is a compilation of these cryophilous species, i.e. those that are present (at least in an ultimate segment of their range) beyond or near the timberline. Pending a thorough investigation of the genus, the above delimitation must be tentative, the more so if the altitudinal as well as the latitudinal timberline is considered.

I have attempted elsewhere (Dansereau, 1955) to outline the geographic extent of these areas in the Northern Hemisphere and to describe the environment as a whole and the peculiar ecosystems that have developed within it.

The principal questions that arise with respect to all and any of these concern phylogeny on the one hand and environmental adjustment on the other. By combining both sets of criteria, relative evaluations of evolutionary opportunity should emerge. Concerning the taxa themselves, the following questions should be posed: What are their affinities? What is their morphology and what local and regional variation do they manifest? What is their genetic balance and degree of isolation? Concerning the total environment which they inhabit, the following points arise: What are their climatic limits? What is their ecological amplitude? What communities are they members of and what is their standing within them?[4]

In this framework, the peculiar cast of each taxon can be outlined and an assessment made of its present evolutionary and ecological status. The taxa can be compared and graded as to:

(*a*) Floristic affinity-group. (Examples: *P. vahliana* belongs to a strictly arctic-alpine complex; *P. arguta* is a member of a very widespread and old cool-temperate flora; *P. anserina* is an almost strictly littoral element with montane extensions.)

[4] I have discussed at length the environmental categories and ecological responses suitable for evolutionary analysis elsewhere (Dansereau, 1952).

TABLE III

A Preliminary List of High Latitude and High Altitude Potentillae
(Wolf, 1908)

Number of Grex	Sp.	Name	Name now used
1	1	fruticosa L.	fruticosa L.
	1a	arbuscula D. Don	arbuscula D. Don
	1b	floribunda Pursh	floribunda Pursh
4	6	biflora Willd.	biflora Willd.
5	9	palustris Scop.	palustris Scop.
6	10	tridentata Sol.	tridentata Sol.
14	42v	arguta Pursh var. convallaria (Rydb.) Th. W.	arguta Pursh ssp. convallaria (Rydb.) Keck
15	52	pulchella R. Br.	pulchella R. Br.
	52	pulchella var. gracilicaulis	glabrella Rydb.?
	53	pseudosericea Rydb.	pseudosericea Rydb.
	54	multifida L.	multifida L.
	65	rubricaulis Lehm.	rubricaulis Lehm.
	75	pensylvanica L.	pensylvanica L.
	76v	glabrella Rydb.	pensylvanica var. glabrata?
16	102	nuttallii Lehm.	gracilis Dougl. ssp. nuttallii (Lehm.) Keck
18	113	nivea L. var. vulgaris Schlecht & Cham.	nivea L. var. nivea
	113v	nivea L. var. pentaphylla Turcz.	furcata Porsild (=quinquefolia Rydb.)
	113v	nivea L. var. uniflora Th. W.	ledebouriana Porsild (=uniflora Ledeb.)
	113v	nivea L. var.?	nivea L. ssp. fallax Porsild (=nipharga Rydb. p.p.)
	113v	nivea L. var.?	sino-nivea Hult.
	113v	nivea L. var. hookeriana Th. W.	nivea L. ssp. hookeriana Hiitonen
	113v	nivea L. var.?	nivea L. var. tomentosa Nilsson-Ehle
	113v	nivea L. var. elongata Th. Wolf p.p.	nervosa Juz.
	113v	nivea L. var. pinnatifida Lehm.?	nivea L. ssp. subquinata (Lange) Hult.
	113v	nivea L. var.?	chamissonis Hult.
	115	villosa Pall.	villosa Pall.
	119	vahliana Lehm.	vahliana Lehm.
19	126	argentea L.	argentea Lehm.
22	181	biennis Greene	biennis Greene
	182	norvegica L.	norvegica L.
	182b	monspeliensis L.	monspeliensis L.
26	244	diversifolia Lehm.	diversifolia Lehm.
27	253	ranunculus Lange	ranunculus Lange
28	265	elegans Cham. & Schlecht.	elegans Cham. & Schlecht.
	266	emarginata Pursh	hyparctica Malte
	272	alpestris Hall. f.	crantzii Beck
30	291	tormentilla Neck. var. typica Th. W.?	erecta (L.) Rausch
31	299	anserina L.	anserina L.
	299v	anserina L. var. egedii Torr. & Gray	egedii Wormskj var. groenlandica (Tratt.) Pol.
	299v	anserina L. var. grandis Torr. & Gray	pacifica Howell

(*b*) Morphogenetic pattern and growth-form. (Examples: the rootstock branching and persistence of dead parts in the various species of the *Niveae*; the shift from palmate to pinnate leaf structure in the *Aureae*.)

(*c*) Genetic lability. (Example: morphologically quite similar plants with different chromosome numbers in the *Argenteae*.)

(*d*) Hybridization with other taxa. (Examples: the more or less indiscriminate crossing in some areas within a grex (*Niveae* in the Arctic) and the occasional crossing between species of very remote sections [*P. pulchella* (*Multifidae*) × *hyparctica* (*Aureae*)].)

(*e*) Ecological amplitude. (Examples: *P. floribunda* is found in many different habitats in different parts of its range, but is rather narrow in any one zone; *P. pectinata* and *anserina* are more or less confined to coastal strands; *P. simplex* ranges from rather shady woodlands and thickets to very open, sunny, windswept areas.)

(*f*) Amount and kind of ecological success. (Examples: *P. nivea* commonly occurs, in the Gaspé Peninsula, as isolated individuals or small, scattered colonies on mountain ledges; *P. norvegica, recta, intermedia* take over old fields by building up extensive and dense colonies. The latter, however, are unstable in time, whereas the former can maintain themselves indefinitely.)

It may be stated, as a preliminary approach to the problem, that the present taxonomic classification of high-northern *Potentillae* is not entirely satisfactory and that the "keying-out" of "species" leaves many units more or less dangling and brings together individuals or populations that are in many ways quite different. In other words, the present hierarchy of taxonomic characters should be questioned, and local (or at least regional) patterns of variation should be investigated and plotted irrespective of their accepted taxonomic allegiance. This is hardly a criticism of the excellent work of taxonomists to date, most of whom are only too willing to submit their determinations to a test.

It is therefore proposed, in the present series of papers:

(1) to review all the available herbarium material and to classify it according to combinations of characters within natural geographical areas;

(2) to present field data collected from populations growing in a natural habitat;

(3) to grow plants of known geographical and ecological origin under uniform conditions and record their morphology and behaviour.

It is hoped that most of the taxa listed in Table III (with possible additions and subtractions) may thus be reviewed and that new taxonomic conclusions can be reached when more is known about their genetic make-up and their ecological integration. For the duration of the investigation, however—especially where (2) and (3) above are concerned—it seems best to retain the accepted taxonomy or to designate the populations by provisional geographic and topographic names. This will avoid any attempt, at first, to decide between a biosystematic and a classical nomenclature, but

may provide a contribution to the vexing problem of their possible or impossible reconciliation (see J. Heslop-Harrison, 1954).

The progress of this project to date, thanks to the co-operation of Dr. Erich E. Steiner and of the University of Michigan Botanical Garden, seems to justify the approach outlined above.

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TRANSACTIONS OF THE ROYAL SOCIETY OF CANADA

VOLUME XLIX : SERIES III : JUNE, 1955

SECTION FIVE

Seasonal Variations in the Resistance of Goldfish to Temperature

WILLIAM S. HOAR

Presented by W. A. CLEMENS, F.R.S.C.

Introduction

SEASONAL variations in thermal tolerance of fishes have been noted (Brett, 1946). These differences have usually been ascribed to acclimatization of the fish to the seasonally changing temperatures of the environment. Less consideration has been given to the possible effect of other environmental factors which vary seasonally. Light, for example, may play a dominant part in the photoperiodic control of endocrine physiology through its effect on the pituitary gland. It is conceivable that metabolism and other aspects of physiology may show seasonal changes which are quite independent of temperature. In fact, it was shown many years ago that the Pacific killifish maintained at constant temperature summer and winter showed a seasonal change in oxygen consumption (Wells, 1935). The physiological basis was not, however, investigated.

During the course of experiments on the effects of dietary factors on thermal tolerance of goldfish, it became apparent that fish maintained and tested under identical conditions of temperature showed seasonal differences in thermal tolerance. In this paper, the pertinent data have been gathered together to substantiate this point.

Several students have been actively engaged in the many experiments from which these data are taken. In particular, F. D. Smith first studied the effects of size on cold tolerance in our laboratory while D. G. Irvine discovered the sex differences and continued the study of seasonal effects first noted in Merva K. Cottle's experiments. Aline B. Redlich, Katherine Newman, and Mary Hollands were responsible for other experiments reported here. It has been a pleasure to have such co-operative and careful students and their contribution is gratefully acknowledged.

Materials and Methods

Goldfish were procured from Stouffville, Ontario, in lots of from 200 to 500 per shipment. They were maintained for variable periods in the laboratory but always for periods considerably longer than required to acclimatize

*This work was supported by grants-in-aid from the National Research Council of Canada.

them (Brett, 1946) to a standard holding temperature of 20°C. Methods for the preparation of the special diets used and for the feeding and care of the goldfish have been described (Hoar and Dorchester, 1949). Treatment with thyroxine, thiourea, or both was carried out by immersing fish in solutions of these drugs. Additional details are given in connection with the particular experiments.

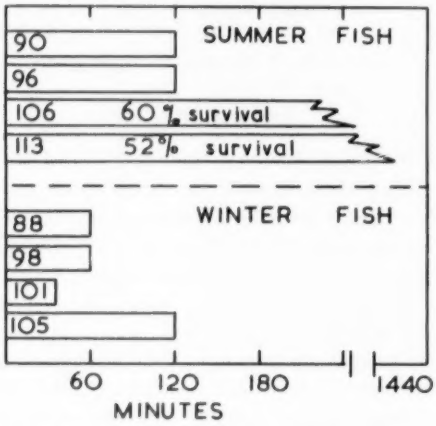

FIGURE 1.—Time for 50 per cent mortality of goldfish placed in water at 36°C. after previous acclimatization to 20°C. and dietary régime of pablum containing 30 per cent hydrogenated pilchard oil; numbers on bars are iodine values of fats extracted from fish; 40–50 fish in each sample tested. Experiments performed by Merva K. Cottle.

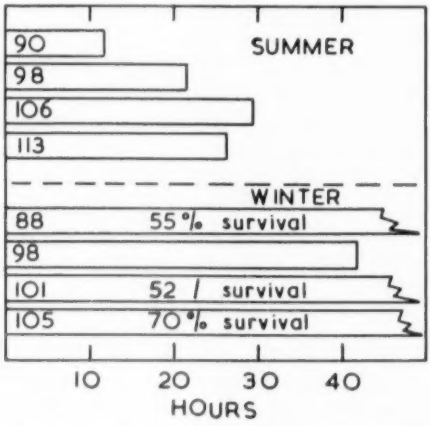

FIGURE 2.—Time for removal of 50 per cent of sample of goldfish tested at 2°C.; experimental procedures as in legend, Figure 1.

The experiments reported here are primarily tests for low-temperature tolerance and were carried out by placing a sample of fish (usually 17 to 20 fish) in a constant temperature bath at 2°C. In the earlier tests (Figs. 1, 2) the temperature was maintained at 2°C. throughout the test. In later experiments the temperature gradually fell from 2°C. to about 1°C. over a period of 5 to 6 hours and was then maintained at the lower level. The heat-tolerance tests reported were performed at a constant temperature of 36°C.

A standard procedure was adopted when removing fish from the low-temperature bath. No fish were removed until one hour had elapsed. Thereafter they were checked at intervals of about one hour. Fish apparently dead were prodded several times at the base of the tail with a glass rod. If there was no response the fish was then removed onto a cold wet glass plate and touched several times with the fingers. If the fish responded on the glass plate it was returned to the low-temperature tank; if not, it was placed in aerated water at about 20°C. Fish removed from the cold tank to the 20°C. bath may recover. These removed fish ("removals") may be either narcotized or dead, and these three categories (removed, narcotized, or dead fish) are considered separately in some of the analyses. Sex and length of the fish were recorded.

Fish referred to as "summer fish" were tested in June, July, or August; those referred to as "winter fish" were tested in November or December.

Contributions of "granulestin" by J. Eichberg, President of the American Lecithin Company, and of pilchard oil by Sven Lassen, Van Camp Sea Foods, California, are gratefully acknowledged.

Results

Seasonal variations in response to temperature

The data in Figures 1 and 2 have been taken from a paper by Hoar and Cottle (1952) and presented in a different form. The fish under investigation had been fed different hydrogenated pilchard oil products in a study of the effects of unsaturation of dietary fat on body fat and temperature tolerance. The seasonal effect is marked. Heat resistance of fish maintained at a constant temperature of 20°C. and tested at 36°C. is markedly greater in the summer than it is in the winter (Fig. 1). Fish fed fats with iodine numbers ranging from 90 to 100 lived about twice as long in the summer. The increased survival was even more marked with the more unsaturated fats (iodine values 105 to 113). In similar tests of cold tolerance (Fig. 2) fish tested in December are consistently more resistant to low temperature than those tested in July.

The fish compared in Figure 3 were fed "pablum" diets or pablum supplemented with 50 percent cholesterol (Merck) or "granulestin" (a mixture of soybean phospholipids prepared by the American Lecithin Company). The particular effects of the fats will be discussed in a later paper. Here it is pointed out that groups of fish with similar diet and thermal treatment behaved differently when tested in the low-temperature bath at different sea-

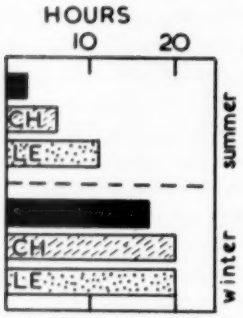

FIGURE 3.—Time for removal of 50 per cent of sample of goldfish tested at 2°C. to 1°C. following acclimatization to 20°C. and diets of pablum (solid bars); or pablum and cholesterol in equal parts (CH); or pablum and "granulestin" in equal parts (LE); feeding period, 20 to 25 days; summer fish tested in June; winter fish tested in November; test samples, 17–20 fish. Data collected by D. G. Irvine.

sons. Survival times were at least twice as long during the winter. In addition, it is apparent (Fig. 3) that the protective effect of the dietary fat supplements is relatively less in winter. It seems that goldfish in winter normally acquire a cold tolerance which is greater than that which can be conferred on "summer" fish by dietary means.

The last comparison is drawn from a series of cold tolerance tests of fish treated with thyroxine (B.D.H. 1:2,500,000) or thiourea (0.03 per cent solution) or mixtures of these materials. Figure 4 is based on fish treated

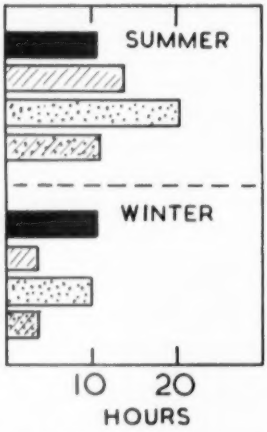

FIGURE 4.—Time for removal of 50 per cent of samples (17–20 fish) tested at 2.0° to 0.5°C. after treatment with thyroxine (cross-hatched) or thiourea (stippled) or both (cross-hatched and stippled); black bars, control fish; previous acclimatization temperature, 20°C.; treatment time, 14 days; diet, pablum. Summer data, July and August, by Aline Redlich; winter data, November, by Katherine Newman.

for 14 days. A consistent pattern of results was obtained with samples of fish treated for 3, 7, 14, and 21 days. The thyroxine-thiourea effect will be discussed in a later paper. The difference in temperature resistance of the treated fish at the two seasons is emphasized here. In the summer, thyroxine increased the survival time by about 30 per cent while thiourea doubled the survival time. In the winter, however, thyroxine and thiourea exert no effect on cold tolerance or are slightly detrimental (Fig. 4). One additional feature is evident in Figure 4. The control fish in summer samples have a greater low-temperature tolerance than most samples of "summer" fish (Fig. 3). In fact the response of the control fish was essentially the same during the two seasons. At least a partial explanation may be offered. The "summer" and "winter" fish were of about the same size but the sex ratio was very different (Table I). Data presented in the next section show that the summer samples with a proportionately larger number of males than the winter samples would be expected to have a relatively greater resistance to cold.

TABLE I

COMPARISON OF MEAN LENGTHS AND SEX RATIOS OF 100 GOLDFISH OF EACH SEX AT EACH SEASON IN 1954

Season	Males		Females		Sex ratio
	Mean mm.	Standard deviation	Mean mm.	Standard deviation	Males/females
July/Aug.	77.4	3.0	77.1	6.4	1:0.91
Nov./Dec.	81.6	9.3	80.3	7.6	1:1.37

Influence of sex and size on thermal tolerance

The data presented above argue for seasonal variations in thermal tolerance of goldfish. Neither acclimatization temperature nor diet seems to be responsible for these differences, since all fish were maintained at 20°C. and since the groups compared received the same diet. However, other sources of variation in the test samples might be responsible for the seasonal differences observed. Gibson (1954), for example, found a difference in the susceptibility of male and female guppies to high temperature while Belehrádek (1935) stated, in his monograph, that age (presumably also size) was the most important internal factor involved in resistance of fish to chilling. In an attempt to discover the basis of the seasonal variability observed in goldfish the possible effect of sex and size differences was examined.

The sex ratios of fish removed from the cold tank are compared in Figure 5. In this graph the observed ratios have been adjusted to show differences in mortality expected in test samples containing equal numbers of male and female fish. It is evident that, during the summer, in the first period of the cold test (period during which 33 per cent of sample was removed) 1.3

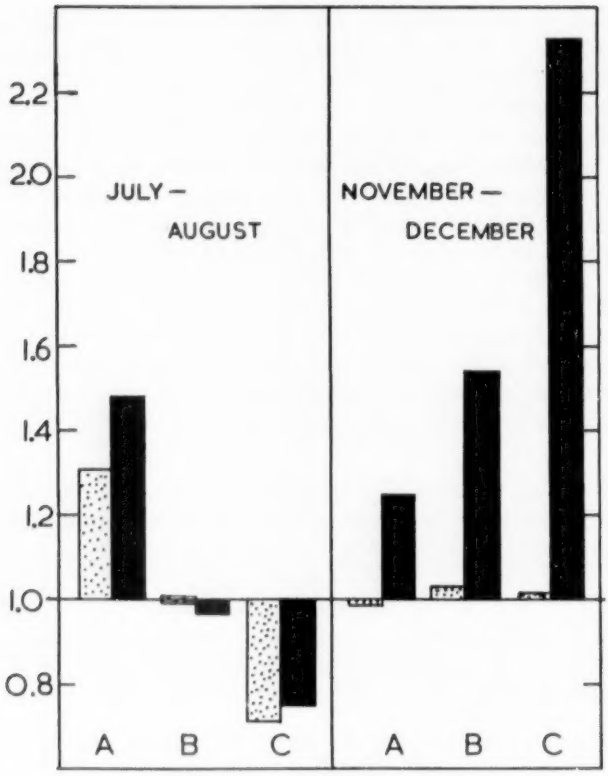

FIGURE 5.—Sex ratios of 20°C. acclimatized goldfish dying from cold of 2°C. during the early (A), middle (B), and terminal (C) part of the cold test. Bars show number of females per one male removed (stippled) or truly dead (black). Observed ratios adjusted to show differences in mortality if equal numbers of males and females had been present in test samples. Histogram based on 524 summer fish and 354 winter fish. Data collected by Irvine, Redlich, and Newman.

females were removed (narcotized or dead) for every 1.0 male removed (narcotized or dead) and of the fish removed during this period there were almost 1.5 truly dead females for every dead male. Since during the summer relatively few fish withstand the cold test, it is obvious that the sex ratios must be reversed at the end of the test after most of the females have died. In any case the greater resistance of males to low temperature is evident. In "winter fish" the picture is quite different since many of the fish tested during this season withstood this test indefinitely. However, the differential susceptibility of the two sexes is again evident. During the early period 1.25 females die for every one male; during the middle part of the test the ratio

has risen to 1.54 females while at the end of the test it has become 2.33 females for each dead male. The sex difference is not marked in terms of removals since the two sexes show about equal tendencies to become unresponsive during the winter. However, many of the males recover from this narcosis while many of the females die.

Since variability of size is inevitable in different samples of goldfish, means and standard deviations of samples of 100 goldfish of each sex were determined for each season. The sexes (Table I) show no statistically significant difference in size at either season although it is evident that the females are on the whole slightly larger. Since larger fish (Table II and Fig. 6) are somewhat more resistant to cold than smaller fish the females have a slight advantage in the cold test. Nevertheless, they die more rapidly, indicating even greater susceptibility of the females than indicated in Figure 5.

The greater resistance to cold on the part of the larger fish is evident in Table II. These data have been compiled from different experiments carried out over a period of three years and involving about 500 goldfish. The pattern is consistent whether considered on the basis of length or weight. The smallest fish are removed (dead or narcotized) during the first part of the test; the largest at the end of the test.

TABLE II

MEAN SIZES OF GOLDFISH (MIXED SEXES) REMOVED EARLY, INTERMEDIATE, AND LATE IN COLD TEST (Irvine, 1954)

Diet	Early period		Middle period		Late period	
	Av. leng.	Av. wt.	Av. leng.	Av. wt.	Av. leng.	Av. wt.
A	72.3	8.75	75.8	10.62	82.2	11.76
B	70.6	8.01	76.0	9.56	79.9	10.39
C	71.8	8.09	80.5	13.48	81.6	11.12
D	74.4	9.22	78.8	11.74	82.1	12.22
P	78.9	10.5	79.9	10.9	82.0	13.3
E	76.6	9.09	80.9	11.7	85.2	13.2
F	80.2	10.7	80.8	11.3	90.0	15.7

The relation between size and survival time is shown more precisely in Figure 6. These data were obtained by cooling goldfish from an acclimatization temperature of 24°C. to 1°-2°C. at the rate of 2°C. per hour. In such a test the cold narcosis is avoided and the fish are truly dead when removed (unresponsive both to mechanical and electrical stimulation in these tests). For each 1.0 cm. increase in length, survival time is increased by about 2.4 hours. A second experiment carried out under comparable conditions gave a regression coefficient of 2.5 hours.

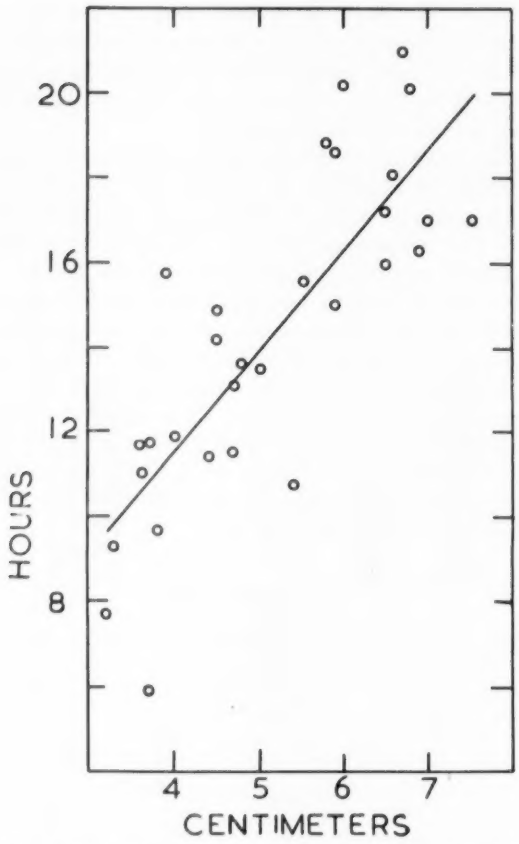

FIGURE 6.—Relation between length of fish of mixed sexes and survival time; 30 individuals tested together at 1°-2°C.; further details in text; regression line, $Y = 1.87 + 2.41X$. Data collected by F. D. Smith.

DISCUSSION

It has been recognized that changes in thermal tolerance of goldfish may be brought about by temperature acclimatization (Brett, 1946) or by feeding different diets (Hoar and Cottle, 1952). In the present study it has been demonstrated that variations in size and in sex may also modify the thermal resistance of goldfish. Seasonal variations in temperature tolerance described above cannot be attributed to acclimatization or diet since these factors were rigidly controlled. Variations in size and in sex ratio did, however, occur in different samples and these factors may have been responsible for some of the differences observed. Size differences, in particular, must be considered since spring and summer growth have produced samples of fish

which are usually slightly larger for the winter tests (Table I). However, several observations indicate that the seasonal differences are not entirely related to increasing size or variable sex ratio but may be attributed to seasonal changes in metabolism and physiology of the organism.

In the first place, relatively few fish of any size or either sex survive the cold test in the summer while many survive in the winter. Thus, of the fish represented in Figure 5, 89.5 per cent of the 524 summer fish were really dead when removed from the bath, while only 26.3 per cent of the 354 winter fish were truly dead. It is evident that winter fish are more often in a state of narcosis when removed in the torpid state from the cold tank while summer fish are more often truly dead. The difference in physiology of the fish at the two seasons is indicated. In the second place the effects of dietary or hormone treatment are quite different in different seasons. The cholesterol/lecithin effect (Fig. 3) is far greater in summer fish and the protective effect of thyroxine or thiourea is evident only in summer fish. This too indicates a real difference in the physiology of the two groups.

Finally, the magnitude of the difference in tolerance of summer and winter fish is greater than would be expected if seasonal growth alone were responsible. On the whole, winter fish are less than 1.0 cm. longer than summer fish (Table I)—usually about 0.5 cm. As shown in Figure 6, the increased tolerance expected on this basis would be from 1 to 2.5 hours. The differences observed in Figures 1-3 are considerably greater. It seems evident that size alone is not responsible for the seasonal variations.

If these arguments are accepted, it follows that temperature tolerance may be added to several other physiological processes known to vary seasonally in fishes. Wells (1935) showed seasonal changes in oxygen consumption of goldfish maintained at constant temperature. Sullivan and Fisher (1953) found seasonal variations in the selected temperatures of trout which were independent of acclimatization temperature. Seasonal changes in endocrine physiology have often been described. Reproductive physiology and growth vary with the season in many fishes, including the goldfish. Other differences have been observed. For example, Müller (1953) described a seasonal variation in the effect of thyroxine or thiouracil on the oxygen consumption of goldfish. Thus, marked seasonal variations in physiology occur and an adaptive seasonal response in temperature tolerance might be anticipated in a fish which must face extreme temperatures in nature.

Light seems to be the most likely environmental variable controlling these physiological cycles occurring in fish maintained at constant temperature. Photoperiodism has been studied in several fish and the observations of Scruggs (1951) on the changing cytological picture of the pituitary in goldfish lends credence to the speculation that seasonal changes in endocrine activity are basically responsible for the differences discussed here.

Moreover, the analysis of the sex data as well as Müller's (1953) work on thyroxine suggest that the problem of seasonally changing thermal tolerance may be a problem of endocrinology. The mechanism is not clear, but

the female fish are consistently less resistant to cold stress. It is interesting that the sex difference appears both in winter fish and in summer fish and thus seems to be independent of the degree of sexual maturity.

In summary, it has been demonstrated that the resistance of goldfish to temperature stress varies seasonally in fish maintained at a constant temperature and fed the same diet. These differences cannot be explained on the basis of variations in sex ratio or in size. It is suggested that photoperiodically controlled changes in endocrine physiology are responsible for these changes in resistance.

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TRANSACTIONS OF THE ROYAL SOCIETY OF CANADA

VOLUME XLIX : SERIES III : JUNE, 1955

SECTION FIVE

Evolution of Meristic Relations in the Dorsal and Anal Fins of Teleost Fishes

C. C. LINDSEY

Presented by W. A. CLEMENS, F.R.S.C.

Introduction

In most animals having a serially repeated row of organs lying along the main body axis, the organs correspond in spacing to segmentation of the body. Typically, one pair of nephridia, one set of muscles, or one set of appendages occupies each body segment.

The median fins of fish form a major exception to this rule. Fin rays and adjacent vertebrae usually do not correspond; instead, the spacing of parts in dorsal and anal fins is frequently quite unrelated to that of the body segments.

The origin of meristic relationships in fish's fins received much attention at the beginning of the present century, for it bore on the origin of tetrapod limbs. The embryology of fins, principally of Elasmobranchs, was hopefully studied in the belief that "ontogeny recapitulates phylogeny." From such investigations, and the then scanty fossil record, the concept was constructed that paired and median fins had evolved from primitively continuous paired and median fin folds in a hypothetical ancestor.

Since that time, more parts of the fossil record have been pieced together. Though prodigious gaps still exist, it is now possible to trace the outline of some groups of fishes as far back as the Devonian era. Since their earliest appearance the bony fishes (Osteichthyes) and the shark-like fishes (Elasmobranchii) have been perfectly distinct from one another. Accordingly, discussion of the probable course of evolution in bony fishes *during the span for which fossil remains are available* cannot validly draw on evidence based on embryology or palaeontology of sharks.

In this paper, meristic relations in the dorsal and anal fins of Teleost fishes and their known ancestors are reviewed. From these data an attempt is made to re-examine the fin fold theory, and to reconstruct the evolutionary changes which have occurred between rays, basals, fin muscles, and adjacent vertebrae in Teleost median fins.

Relevance of Non-Teleost Fishes

Early discussions of the origin of paired and median fins of fish assumed or implied a fairly close line of descent between sharks and modern bony

fishes. The mass of palaeontological data accumulated in the last few decades has tended to discredit this view. It now appears that the major groups of modern fishes have been evolving along separate lines for a very long time; Cyclostomes, Elasmobranchs, Chimaeras, Dipnoans, and Actinopterygians have probably been distinct from each other since Devonian times (19). Therefore certain fossil forms formerly held to support the fin fold theory must be discarded as irrelevant to the question of fin evolution in the ray-finned fishes, and even in modern sharks. Fossil Acanthodian sharks, whose series of paired spines were thought to represent the breaking up of paired fin folds, have now been clearly shown to have originated from quite different ancestral types than have modern sharks (24). Similarly the Devonian shark *Cladoselache*, with wide-based fins suggesting fin folds, cannot now be considered as ancestral to modern Elasmobranchs, much less to modern Teleosts (19).

Some recent authors have turned to the agnathous Ostracoderms, presumably ancestral to all higher vertebrates, in an attempt to trace the evolution of fins. Eaton (6), who stated that palaeontological support for the fin fold theory has been largely if not wholly eliminated, concluded that fins arose as, or in conjunction with, median and paired spines in Ostracoderms. Gregory and Raven (12) suggest that at least the pectoral girdles of all later fishes were derived from the shields of Pteraspid Ostracoderms.

These theories implying single origin of all vertebrate fins from median and paired spines in Ostracoderms may be oversimplifications, for apparently fins have arisen independently in several different groups during the course of evolution. Westoll (24) presents evidence that mobile paired and median fins have arisen from stiff bone-jacketed structures independently in Acanthodian sharks and other Placoderms, and independently at least twice in the latter group. Further, among Ostracoderms, paired fin-like structures have apparently arisen independently at least twice in Cephalaspids and Anaspids, and not at all in Pteraspids.

Mention should be made of the Silurian fossil *Jamoytius kerwoodi*, perhaps the most primitive vertebrate so far discovered (26). This form may have possessed paired and median fin folds. Its relationship to other vertebrates is obscure, but it suggests that fin folds were perhaps evolved in some groups prior to the origin of the earliest members of those groups which can presently be identified.

In discussing *Jamoytius*, White (26) observes that undue stress may have been laid on the supposed loss of armour during early fish evolution. Unossified forms may have lived side by side with armoured fishes but have been preserved only rarely. Cyclostomes may not be degenerate descendents of Ostracoderms but may instead preserve some characteristics of a remote unossified ancestor. Indeed, known Ostracoderms may have already diverged widely from the ancestor they share with higher fishes.

No single theory of the origin of fins is likely to encompass all the vicissitudes of these variable structures. Lacking detailed knowledge of the stem

forms from which different major groups of fishes have diverged, we must at present consider evolution within each group separately. Discussion will here be confined to the ray-finned fishes, a group whose ancestry is traceable at least in broad outline as far back as the Middle Devonian.

Teleost Ancestors

Before discussion of modern Teleost fishes, the meristic relations between rays, basals, and axial skeleton in ancestors to the Teleosts will be outlined. Only the ray-finned fishes (Actinopterygii) are considered. Known Dipnoans and Crossopterygians, whose median fin structures are unlike those of Teleosts, can no longer be considered as ancestral to the latter. Ray-finned fishes were already widely divergent from the other two groups in their earliest known representatives, judging from such features as fin structure, scale structure, and absence of internal nostrils (19).

Eaton (6) standardizes the terminology of median fin supports. The internal cartilage or bone on the distal end of which the dermal fin ray articulates is a "pterygiophore." Each pterygiophore may develop as a series of two or three cartilages, the "radials." Often the proximal radial is notably larger than the middle and distal radials; it is then spoken of as a "basal." The terms "baseost," "axonost," "interneural," and "interhaemal" are to be avoided.

Probably ancestral to all modern Actinopterygians were the Palaeoniscoidea. These comprised a large group of Chondrostean fishes which first appear in Devonian rocks. In early Palaeoniscids the external dorsal and anal fin rays greatly exceeded the number of "basals" or internal skeletal supports (Fig. 1A). A reduction in the number of rays per basal took place during the evolution of the group, but the few forms surviving beyond the Palaeozoic still maintained an excess of rays over basals. The modern sturgeons and paddlefishes, contemporary Chondrosteans, also maintain this condition. In the dorsal and anal fins of *Acipenser* (Fig. 1B) the fin rays outnumber the basal supports, and the spacing of the rays and basals bear no simple relation to each other. *Polypterus* maintains the Chondrostean type of anal fin with more rays than basals, but the dorsal is aberrantly modified into a series of small "sails."

An assemblage of fishes known collectively as Subholosteans was derived from Palaeoniscids in the Permian and flourished during the Triassic. Most Subholosteans showed a further reduction in the number of rays in both paired and unpaired fins, so that the external rays coincided in number and spacing with the endoskeletal supports. An intermediate condition occurred in some families; in Pholidopleuridae and Cleithrolepidae the rays corresponded to the basals at the posterior but still outnumbered them at the anterior of both dorsal and anal fins (3).

The next composite group, the Holosteans, abounded in the Mesozoic era and survive today as the garpike *Lepisosteus* and the bowfin *Amia*. In all these and in their successors, the Teleosteans, which dominate the recent

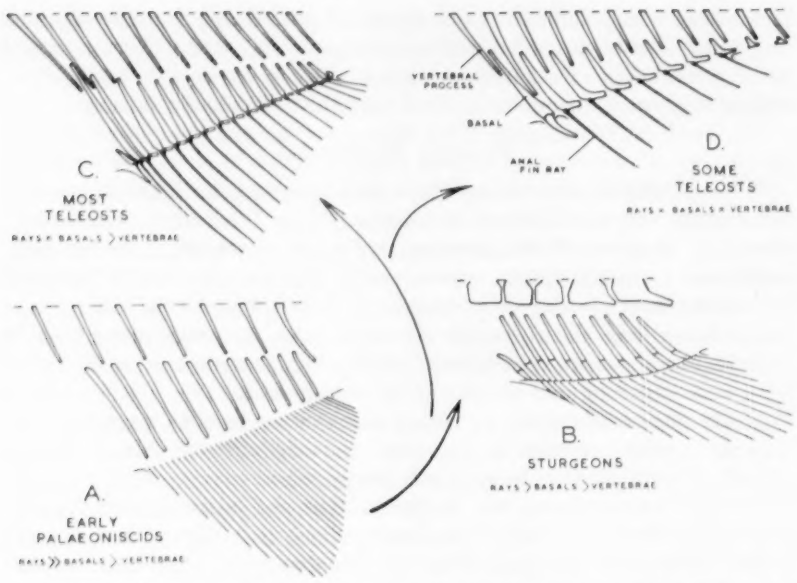

FIGURE 1.—Meristic relationships in the anal fins of representative Actinopterygian fishes. Branching of fin rays omitted. A. *Carbovales ovensi* White, Lower Carboniferous (after White, E. I., 1927); B. *Acipenser ruthenus* L. (after Goodrich, E. S., 1909); C. *Richardsonius balteatus* (Richardson); D. *Gasterosteus aculeatus* L.

fish fauna, the median fin rays correspond one-to-one with the underlying basals at all but the extremities of the fin (Fig. 1C, D).

Decrease in fin ray number per unit body length in Holostei and Teleostei has resulted, on the average, in an increase in the space between successive rays. The middle and distal elements of each pterygiophore are inclined backwards, the middle one, if present, forming a "spacer" between the distal extremities of successive pterygiophores (Fig. 1C). The middle radial may be lost, but the distal radial is always shifted backwards so that the ray it supports comes to lie in line with the next posterior basal (Fig. 2). The wide membrane connecting adjoining rays may be strengthened by distal branching of each ray.

Relations between the endoskeletal supports of dorsal and anal fins and the vertebrae do not follow so clear an evolutionary progression. The basal elements immediately under the external rays were more numerous than the adjacent vertebrae in Palaeoniscids (Fig. 1A). This condition persisted in the majority of later Chondrosteans and Holosteans (Fig. 1B). The greater part of Teleosts also have more median fin supports than adjacent vertebrae (Fig. 1C), but a certain proportion have developed a one-to-one ratio between basals and vertebrae (Fig. 1D). The phenomenon occurs sporadically among the higher groups of Teleosts, and is apparently secondarily acquired rather than retained from a primitive state.

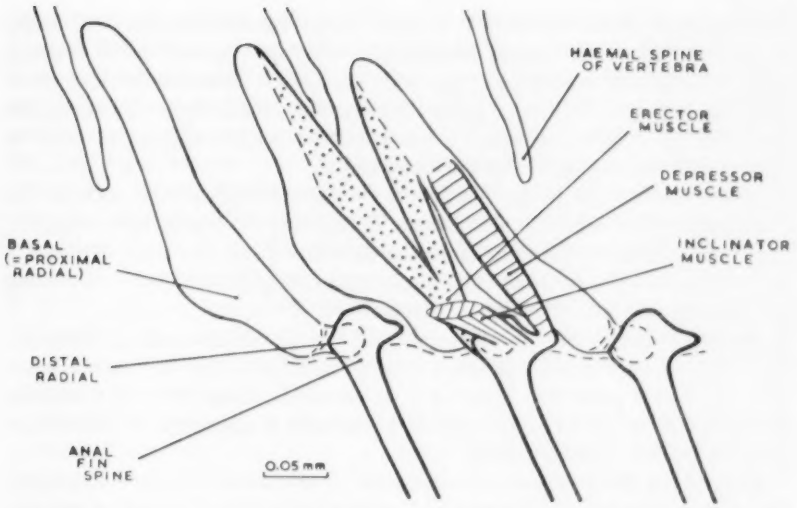

FIGURE 2.—Portion of base of developing anal fin, *Macropodus opercularis*, viewed from left side. Only one set of fin muscles is shown. Reconstructed from sagittal sections of 8.0 mm. larva.

The median fin supports are in many cases biserial or triserial with numerical correspondence between the proximal, middle, and distal series of elements. In some Palaeozoic Palaeoniscids (*Dorypterus, Bobasatrania*) an inner series of detached median elements lay within the outer series and corresponded in number more closely to the vertebrae than to the outer series. In Jurassic Palaeoniscids this inner series was absent, leaving a single row of basal elements outnumbering the adjacent vertebral segments (23). The relationships of the inner series are obscure, and White (25) believes that *Dorypterus* and *Bobasatrania* occupy isolated positions away from the main evolutionary trends.

MODERN TELEOSTS

A typical modern Teleost pterygiophore includes a large basal which may extend inwards almost to the vertebral column. (In the sea-horse *Hippocampus* the basals are secondarily fused with the vertebrae.) The shorter middle radial slopes backwards from the distal end of the basal; at its posterior extremity lies the small distal radial, which overlies the basal of the next posterior pterygiophore. Many variations of this triserial plan occur (2). The pterygiophores of spines are composed of only proximal and distal radials, or merely proximal radials. Sometimes (Blenniidae, Labridae) the middle and distal elements are missing, and the rays rest solely on the underlying basals. Proximal and middle radials ossify if present, but the distals remain cartilaginous.

Support of the fin membrane is provided by dermal "fin rays" (flexible, marked by striae, and usually branching) or dermal "spines" (stiff, without striae). Regularly spaced rings mark the ray shaft. Towards the fin's outer edge the ray may bifurcate antero-posteriorly several times. Towards the base the ray is split sagittally; the two halves are laterally compressed to form a pair of tongs grasping the distal radial.

Numerous slender horny fibrils, the "actinotrichia," may persist in the outer edge of the adult fin membrane. These were functional as strengtheners in the embryonic fin fold before the definitive rays (the "lepidotrichia") were formed. Their number is indefinite and they probably have no bearing on meristic relations of adult fin structures.

Fin spines follow the same plan as rays, but lack both striae and branching. They are stiff and often sharp pointed. Unlike the fin ray, which has a ball and socket joint that allows side to side undulation of the soft fin, the spines may be hinged to allow movement only in a sagittal plane. Inclinator muscles are sometimes lacking.

Despite the wide variation in structure of dermal elements, endoskeletal supports, and muscles of Teleost median fins, the serial relationships between these three series are constant. Except for departures at the extreme ends of the series (commonly arising from fusion of the anterior basals [2]), there is one-to-one correspondence throughout; each ray or spine is supported by one basal, and operated by one set of muscles.

While dermal and endoskeletal fin elements are in step where the two are coextensive, there are a few cases of basal series extending beyond the overlying rays or spines. Mohr (17) describes pterygiophores with no corresponding rays lying between the two dorsal fins of the mackerel. In the swordfish, according to Mohr, the second dorsal and second anal fins are absent, but reduced pterygiophores remain. In the paradise fish (15) a few spineless basals lie anterior to and continuous with the basal series of the spinous dorsal. In three species of sticklebacks (*Gasterosteus aculeatus* L., *Eucalia inconstans* (Kirtland) and *Pungitius pungitius* (L.)) the pterygiophore series extends several segments posterior to the last fin ray in both dorsal and anal fins.

The foregoing examples of rayless or spineless ptergyiophores represent structures similar to and homologous with ray- or spine-bearing pterygiophores. A problematical series of median detached rods occurs anterior to the first dorsal fin in many of the lower Teleosts. These "supraneurals" usually correspond one-to-one with the neural vertebral processes. The supraneural series may be continuous with the dorsal fin pterygiophores, or separated from them by a gap. In some fossils the two series overlap (e.g. *Leptolepis, Thaumaturus*). Supraneurals are not necessarily homologous in all fish; they have been variously interpreted as detached vertebral processes and as rayless pterygiophores (6; 11).

The common lack of serial correspondence between median fins and trunk segments has been mentioned. From one to six or more median fin

rays, with a corresponding number of basal elements, may occupy one body segment in Teleosts. Fin rays do not normally occur without basals, nor are they spaced more widely than one per body segment. One ray and basal may occupy each segment along almost the entire length of the vertebral column, or along any shorter portion thereof. (For example the scabbard fish *Lepidopus peronii* has 103 dorsal rays each adjacent to one vertebra [1].) More frequently the number of rays is higher than the number of adjacent vertebrae. Ray counts may reach very high numbers; the oarfish *Regalecus argenteus* has 93 vertebrae and 205 dorsal rays (18), while the gymnotid *Rhamphichthys rostratus* may have up to 515 anal fin rays (7). A further case of independence of fin rays and vertebrae is found in the ocean sunfishes (Molidae) in which the posterior few rays and basals of both dorsal and anal fins are complete although the corresponding axial segments have atrophied (9).

In all these cases where the rays and vertebrae do not correspond, each of the axial and fin series is evenly spaced within itself. The two series are apparently quite independent of one another at their earliest appearance in the larva. (See for example figures of young *Solea vulgaris* by Fabre-Domergue and Biétrix [8].)

The remaining fins of Teleosts will be omitted from this discussion of meristic relationships. The adipose fin has no internal skeletal supports and only an indefinite number of horny actinotrichia in the fin membrane. Muscles are absent from the adipose except, rarely, in the form of an inclinator layer inserted on a membrane in the mid line (13).

In the caudal fin there is no serial correspondence between dermal rays and internal skeletal elements; the former always outnumber the latter. The ventral lobe of the tail fin is supported by bones probably homologous with haemal vertebral processes, while the dorsal lobe may contain modified neural processes, or modified pterygiophores, or both. The degree of fusion between these various elements differs greatly phylogenetically, as does the caudal fin musculature.

In the paired fins the radial skeletal supports are in most cases also outnumbered by the dermal rays. Certain trunk segments can be related to the pectoral or pelvic fins by tracing those spinal nerves which contribute to the limb nerve plexus, but no serial correspondence appears between spinal nerves, radial supports, and dermal rays.

Median Fin Muscles

Teleost median fin rays and spines are typically each provided with three pairs of muscles, one of each pair being inserted on each half of the ray base. On each side, a depressor muscle (Fig. 2) is inserted on a posterior projection of the ray base. This muscle originates from the anterior or the lateral face of the next basal towards the posterior (i.e., that basal which in the adult lies with its longest arm in line with the ray). Contraction of

this muscle pair serves to rotate the ray about the centre of the distal radial and hence to depress the ray (Fig. 2).

An erector muscle is inserted on a projection from the anterior of the ray base. Its point of origin is variable. In some fishes (e.g. *Amia, Gadus, Gasterosteus*) the erector muscles originate from the posterior of the basal initially associated with that ray, or from a membrane in the mid line connecting this basal to the next one posterior. In others the erectors originate partly or perhaps wholly from the anterior of the next basal behind (i.e., from the same basal as the depressor muscles of that ray). An intermediate condition occurs in young *Macropodus* (Fig. 2), where the erectors are clearly separated into two parts, one running to the associated basal and one to the next basal posterior.

The inclinator muscles are inserted on the sides of the ray bases (Fig. 2) or sometimes on the skin between rays. They run obliquely forward and outward and usually originate between the skin and the upper surface of the lateral body musculature. In many lower Teleosts (e.g. Cyprinids) the whole of the fin base stands out from the body; here the inclinators originate from the outer faces of the erector and depressor muscles.

The points of origin and insertion of all these muscles are subject to variation which has been described by Grenholm (13). The erectors and depressors may sometimes serve to incline the rays, a point of importance in considering the evolution of these muscles.

Nervous supply to the dorsal fin muscles is from the spinal nerves of body segments lying adjacent to the fin base. These anastomose and are joined by branches from the *ramus lateralis trigemini* (16; 22). The whole forms a longitudinal collector along either side of the base of the fin muscles. From this plexus separate branches run to each set of fin muscles. In the anal fin a collector is also formed from branches of the adjacent spinal nerves.

Nervous supply to particular fin muscles cannot usually be allocated to particular spinal nerves. Even in *Gasterosteus aculeatus*, in which a single set of fin muscles lies opposite each vertebral segment, branches of the spinal nerves anastomose to form a longitudinal collector.

The descriptions above apply to Teleosts in which each ray corresponds to one basal and is operated by one set of muscles. In modern Chondrosteans the closely set rays allow of little antero-posterior motion. Muscles arising from one basal must be shared by several rays. In the paddlefish *Polyodon* there are but two types of indistinctly separated fin muscles, both of which act largely as inclinators (4). Dissection of *Acipenser transmontanus* also shows muscles indistinctly separated into inner and outer layers; muscles arising from the lateral face of one basal are inserted on the bases of two or three adjacent rays and on the surrounding skin. In *Acipenser* the pterygiophores extend out between the ray bases well beyond the body wall. (The distal and part of the middle radials are hidden in Fig. 1B by the rays.) The joint between proximal and middle elements evidently contributes some flexibility to the fin base. When the fin is inclined some flexion occurs be-

tween the rays and pterygiophores, and some within the pterygiophores between proximal and middle elements.

Teleost ancestors, the Palaeoniscids, also had a large excess of rays over basals. Because of the closely packed rays, little elevation or depression of the fin can have been possible in early forms (Fig. 1A). Principal muscular control must therefore have been provided by inclinators, although these were perhaps not homologous with Teleost inclinator muscles. If we suppose that Palaeoniscids had fin musculature similar to *Acipenser*, then Palaeoniscid muscles originating from the basals but serving as inclinators have probably been transformed into the erectors and depressors of Teleosts. The inclinator muscles in modern Teleosts, originating from the skin, are then a new development.

There is also the possibility that muscles operating the Palaeoniscid median fins had their origin on the fascia of the body muscles just as do the inclinators of Teleosts. The pterygiophores in early Palaeoniscids or their ancestors did not necessarily serve from the first as points of muscular attachment. In Teleosts, basals without rays, supraneurals, intermuscular bones, and, in Clupeidae, large median scutes, all serve as internal strengtheners. Perhaps in Palaeoniscids the basals underlying (and outnumbered by) the fin rays first evolved as mechanical supports, and did not serve until later as points of muscular attachment. Muscles readily shift their points of origin; median fin muscles have in various Teleosts seized upon the skull, the cleithrum, and the vertebrae as points of attachment (13). Therefore the muscles which today arise from the basals may originally have arisen from the body muscles. With all these possibilities, homology and analogy are inextricable.

A further set of muscles should be mentioned in connection with the median fins. The supracarinales are narrow muscles running on either side of the mid-line from the head to the anterior of the dorsal fin, and from the posterior of the dorsal to the caudal. Comparable infracarinales run from the cleithrum to the pelvic fins, from the pelvics to the anal fin, and from the posterior end of the anal to the caudal. These muscles are attached to the basals of the dorsal and anal fins, and do not take part in moving the rays. They are probably homologous with the fin muscles, for they arise in the same way from the myotomes and may display segmentation independent of the body. This homology will be apparent from the following discussion of fin muscle development. Carinal muscles have been variously interpreted as vestiges of a longer series of fin muscles and as the primordium from which fin muscles have evolved (13).

Embryonic Merism

The following section outlines the embryology of Teleost median fins, a subject which will be treated more fully in another paper. Median fin development is externally similar in most Teleosts. In the embryos of all but a few species a median fold of epidermis runs continuously from the anus

back around the tip of the tail and forward dorsally to a point near the head. Into this epidermal fold, mesenchyme insinuates to form local thickenings at the sites of the dorsal, caudal, and anal fins. Subsequently the intervening parts of the fold disappear.

Internally, the rays, basals, and median fin muscles arise from different embryonic tissues. Fin rays arise from the basement membrane of the dermis, or, doubtfully, by fusion of fibrils internal to the basement membrane. Fin rays appear embryonically in their definitive positions, with their relationship to the underlying basals fixed from the first. This is true both in Teleosts, where rays conform serially to basals, and in *Acipenser*, where they do not. Fin ray formation has obvious similarities to scale formation, as the name lepidotrichia implies. Some Palaeoniscids had rows of scales applied to the outsides of the fin rays and continuous with the body scales (6).

The internal skeletal supports or pterygiophores of the median fins arise as a series of local differentiations in mesenchymatous connective tissue filling the embryonic fin fold. "Although probably this tissue has itself been derived from segmental sclerotome, yet the limits of the segments have long ceased to exist when the radials develop" (10). As with fin rays, meristic relations of the pterygiophores are fixed from their earliest embryonic appearance. Embryological evidence does not suggest that pterygiophores represent pieces detached one from each vertebral process and subsequently converging under the fin base.

Muscles of Teleost median fins arise from proliferations from the tips of adjacent myotomes. In the unpaired fins of the primitive forms, *Acipenser*, *Lepisosteus*, and *Amia*, distinct drop-shaped muscle buds are formed one from each myotome involved; these appear to "converge" or lean in towards the site of the definitive fin base (21). Each pair of buds separates from its parent somite and forms the muscles for one median fin ray, except in *Acipenser*, where the rays are more numerous and one set of muscles is inserted on two or three adjacent rays.

In *Salmo salar* drop-shaped muscle buds are also given off by the somites, but the number of pairs of buds is less than the final number of sets of fin muscles; the additional members of the series are formed anterior to the first muscle buds by successive appearance, in the mesenchyme, of pterygiophores alternating with muscles (14). This mode of muscle formation is intermediate between that of Chondrosteans and Holosteans and that of certain higher Teleosts. In most of the latter which have been examined, no discrete buds arise from the myotomes. Instead, loose proliferations of amorphous material from the myotome tips aggregate along the fin base to form a strip which subsequently gives rise to fin muscles with spacing quite independent from that of the parent somites.

To summarize, the rays and pterygiophores arise *in situ* with their serial relations determined from the first. This is true also of the fin muscles in higher Teleosts, but in lower bony fish the myotomes contribute discrete buds which appear to converge to some extent before reaching their defini-

tive positions. The significance of this phenomenon of convergence is discussed in the following section.

Fin Fold Theory

In past discussions centred around the "fin fold theory," the phrase has not always had the same meaning. Discussion which roused the most interest concerned the origin of the *paired* fins, for this question had bearing on the origin of tetrapod limbs. The fin fold theory as applied to paired fins stated that these fins are derived phylogenetically from the paired longitudinal fin folds of a hypothetical ancestor. The main rival theory, attributed to Gegenbauer, supposed that paired fins are modified gill arches. The latter theory has been discredited on several grounds. The fin fold theory of the origin of paired limbs has much in its favour, but it does not stand automatically proven by the fall of the gill arch theory. Alternative theories such as origin of fins from paired spines have been advanced (6), and the question is still open.

A quite separate fin fold theory concerns the origin of the *median* fins of fishes. Most authors, even those who were opponents of the paired fin fold theory, have agreed that the dorsal, caudal, and anal fins must have arisen by the break-up of a primitively continuous median fin. In opposition to this theory Eaton (6) suggests that median fins are derived from median spines in Ostracoderms. For reasons stated earlier those portions of his evidence drawn from non-Actinopterygians are excluded from this discussion of Teleost evolution. Eaton's interpretations of muscle-bud convergence and of Teleost embryonic fin folds are largely in accordance with the following.

The "convergent" appearance of muscle buds leaning towards the site of the fin base in primitive Teleosts, as well as the comparable convergence of nerves in the adult fins, led authors in the past to suppose that actual convergence of fin elements has occurred in the course of evolution (11). Starting with a hypothetical ancestral adult in which one basal lay opposite each somite and received one set of muscle buds and nerves from it, the basal elements are supposed to have moved together to their present condensed condition, carrying their muscle buds and nervous supply with them.

Little support for this hypothesis of convergence has been found in the fossil record of Actinopterygians. Basals have outnumbered adjacent vertebrae since the earliest Palaeoniscids, and basals correspond serially to vertebrae only in sporadic groups which according to palaeontological evidence have attained this condition secondarily. There is, therefore, no palaeontological suggestion that basals of the dorsal and anal fins represent detached portions of the vertebral column (although such may be the case for certain caudal elements and perhaps certain pre-dorsal "supraneurals").

An alternative interpretation, and one consistent with the available evidence, follows the more recent concept that the embryology of modern fish repeats not the adult but the embryonic form of their ancestors. Convergence

of muscle buds and nerves may have occurred in the embryonic fin development of early Palaeoniscids much as it does in some of their living descendents.

Part at least of the slope of each outlying muscle bud towards the centre of the developing fin may be due to differences in growth rates of the fin base and of the body axis. Eaton (6) attributes the whole of the "illusion" of convergence of muscle buds to faster growth of the body. Although the convergent effect in muscle buds may well be attributable to growth differences, such differences have yet to be measured. The leaning together of buds in Schmalhausen's figures of *Amia* and *Lepisosteus* is pronounced; a large difference in the rates of elongation of body and fin base would have to be demonstrated in order to establish that this was the sole factor responsible for convergence in these fish.

Centres of active differentation in embryos have been shown by experimental embryologists to be capable of attracting developing nerves. Such attraction offers an alternative explanation of nerve convergence in embryonic fins, without invoking the theory of recapitulation. When amphibian limbs have been experimentally transplanted, brachial nerves have been attracted to the new position (5). Orientation of nerves during growth has been shown by *in vitro* experiments to be governed simply by mechanical stresses in the surrounding medium (5). Thus, the apparent faithfulness of a nerve to its muscle segment (10) has been shown to be fickle, and we need not read too deep a meaning into the congregation of nerve-endings in a developing fish fin.

Another feature of Teleost development which has been interpreted as embryonic recapitulation is the occurrence, in the larval stages of most species, of a continuous median fin which subsequently breaks up into dorsal, caudal, and anal fins. This larval fin fold is almost certainly functional. Before the definitive paired and median fins are formed the continuous fin has an important locomotory function for the young fish. Amphibian larvae also possess a median fin fold before their paired limbs develop. Indeed, the three Teleosts listed by Ryder (20) which lack continuous embryonic median fins (*Gambusia*, *Siphostoma*, and *Hippocampus*) are all viviparous species in which the young lack a protracted free-swimming larval stage and hence have no need for a continuous fin fold. In other fish and in amphibians the larval fin fold has definite if transitory functional advantage; therefore the fold need not be interpreted as an evolutionary relic.

It must be concluded that the evidence formerly advanced in support of the theory of the origin of Teleost median fins from a primitively continuous fin fold is far from conclusive. Palaeontological evidence is to the contrary and embryonic patterns have received alternative explanations.

That is not to say that the fin fold theory has been disproven. New palaeontological evidence may yet show that ancestors of Palaeoniscids were, like *Jamoytius*, unossified and possessed of a continuous median fin membrane.

On the other hand, they may have been Ostracoderms, whose spines gave rise by multiplication to later median fins. At present we may state that nothing in the evidence suggests evolution from a fin fold after the first known Palaeoniscid. As to what occurred before then, decision must wait upon the discovery of more pre-carboniferous fossil fishes.

Evolution of Serial Correspondence

In adult Teleosts the functional co-ordination between each ray and its basal and muscles might suggest that perfect serial correspondence must have existed between the three units throughout their evolution. Yet such is certainly not the case. Vertebrae, internal basals, and external median-fin rays were serially independent of one another in the earliest known ancestral Teleosts. A gradual convergence towards serial correspondence occurred between the latter two series and, in some groups of fishes, amongst all three series.

One-to-one correspondence between fin rays, muscles, and basals (and in some cases also vertebrae) has probably distinct mechanical advantages, accounting for the almost universal occurrence of the plan among surviving Teleosts. In early Palaeoniscids with close-packed rays, the fin movement was probably provided largely by inclinator muscles. Full development of erector and depressor muscles, one set originating from each basal, probably occurred when the number of fin rays was reduced to the point where each ray articulated with a single underlying basal.

Phylogenetic reduction in number of fin rays (relative to basals) probably occurred through progressive strengthening of inductive mechanisms which produce a single ray opposite each basal. Judging from the intermediate condition in some Subholosteans, this occurred first at the posterior end of the median fins, in that section of the fin which in modern fish is last-formed embryonically and is most readily alterable experimentally. This pattern in median fins of a rigid anterior and mobile posterior end seems to conform to the hydrodynamic requirements of fish. In surviving Chondrosteans, rays still outnumber basals along the whole of the median fins, but it is the posterior rays which are capable of the greatest mobility (4). In most contemporary fish, maximum flexibility of the median fins is found at the posterior end, while the anterior is frequently modified into a rigid keel.

Serial correspondence between basals and rays has allowed specialization of different parts of the median fins. In higher fish, the anterior spinous dorsal can be erected rigidly for stabilization, defence, or display, and it can be folded flat. The posterior soft dorsal is a versatile propellor, with each ray moving on a universal joint and operated by three sets of muscles. The whole represents a great mechanical advance over the first Palaeoniscid median fins, which must have been limited largely to lateral undulation and incapable of retraction.

The basic plan of serially repeated rays and basals has allowed spectacular variation in number and nature of fin parts. These larger variations on the

general theme are presumably adapted to such individual requirements as speed, manoeuvrability, defence, and display. Adaptive radiation in the median fins has been largely dependent on the evolution of serial conformity between fin rays and their internal supports.

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TRANSACTIONS OF THE ROYAL SOCIETY OF CANADA

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SECTION FIVE

Range of Structural Variation in Organic Terrain

NORMAN W. RADFORTH

Presented by GLEB KROTKOV, F.R.S.C.

Introduction

FROM the aspect of national economy, problems concerning organic terrain in Canada are now becoming very critical indeed. The development of the Northwest in particular is accompanied by heavy financial loss because the properties of muskeg are seriously inhibiting normal engineering progress. The frustration arising from this is intensified by the obvious fact that the rate of development of northern exploration is ever increasing, and it has surpassed the contribution that muskeg research has so far made. The concern shown by the oil and forestry industries and by agencies responsible for road construction is being brought to the attention of the Associate Committee on Soil and Snow Mechanics of the National Research Council. It is hoped that this trend will continue, for it effectively leads to formulation of pertinent programmes for muskeg research.

For some years the writer and his associates have carried on muskeg investigation under the sponsorship of the Associate Committee on Soil and Snow Mechanics and the Defence Research Board. One aspect of the work has centred around ground studies (2, 3, 4), the other on aerial interpretation (5). At the present stage of progress, to satisfy both interests and to contribute towards the solving of the pressing engineering requirement, there is need to fulfil a particular subsidiary objective in the programme: the establishment of a classification system for macroscopic constituents of the peaty matrix of the organic terrain.

In the present work the writer wishes to assess the limitations of this system and test its application.

Macrosopic Structural Entities

In another account reference has been made to 16 categories of peaty structure which are encountered in the composition of organic terrain (5). For convenience here, these are again listed (Table I), and in the same order. Another useful set of categories has been supplied by Dachnowski-Stokes (1) in his work on southern peats. A list of his categories with slight modifications in arrangement appears in Table II.

The degree of contrast between the two sets of categories (cf. Tables I and II) will be readily appreciated. The large number of entities in the one

as compared with the other is marked. The conceptions of form suggested by the two sets of designants differ widely. Interpretation of constituents as represented by the nomenclature differs somewhat in the two cases. Size of primary constituents has also been viewed differently. Combination refer-

TABLE I

DESCRIPTIVE TERMS FOR SIXTEEN CATEGORIES OF PEAT STRUCTURE

Category number	Description
1	amorphous-granular
2	non-woody fine-fibrous
3	amorphous-granular in fine-fibrous
4	amorphous granular in woody fine-fibrous
5	amorphous-granular, fine-fibrous, non-woody in woody fine-fibrous
6	amorphous-granular with woody fine-fibrous held in coarse-fibrous
7	non-woody fine-fibrous covering amorphous-granular in fine-fibrous
8	non-woody fine-fibrous; mound in coarse-fibrous
9	woody fine-fibrous held in coarse-fibrous
10	woody particles in non-woody fine-fibrous
11	woody and non-woody particles in fine-fibrous
12	coarse-fibrous, woody
13	coarse-fibrous traversing fine fibrous
14	non-woody and woody fine-fibrous held in coarse-fibrous
15	woody with amorphous-granular in fine-fibrous
16	woody coarse-fibrous with scattered woody erratics

TABLE II

TERMINOLOGY CATEGORIZING STRUCTURE IN PEAT DERIVED FROM DACHNOWSKI-STOKES DESCRIPTION

Plant source	Peat type	Texture
Macerated Colloidal	pulpy	coarse to very finely divided
Reed Sedge Brown Moss Bog Moss Heath Shrub	fibrous	coarse to fine-fibrous
Trees	woody	woody

NOTE: Structure in this system is referred to in terms of properties of identified units, e.g. compact, dense, matted, etc., and thus does not parallel the interpretation of structure by the present writer.

ence entities in one set emphasize meanings that either do not apply for the other set or apply in another way. Finally, although generic implications have not been avoided in either list, categories in Table I emphasize physical aspects of structure while those in Table II relate more directly to botanical structure.

To proceed at further length with the comparison would hardly be justified. Dachnowski-Stokes in his analysis of macroscopic detail was presumably concerned with sequence of materials to show how plant organisms *in toto* contributed to the bulk in peat constitution. In this he succeeded admirably. His contribution has also been very useful in establishing as a principle the conception of gross compositional change on the botanical basis. The work provided the fundamentals of a reference method for those in the applied field who wish to make a quick and valid assessment of mass structure.

The requirements with which the writer has had to deal are beset by other specifications sometimes more exacting. In previous work the reasons for utilizing the components of the 16 categories (Table I) are not given. Variability in vehicular response to change of organic terrain was one circumstance which had a relation to the terms for selection of macroscopic reference-matter. Degree of resistance to vertical compressional forces was another. Stability of the organic medium when subjected to mechanical forces was a third. Some others are relative permeability of the organic medium, insulation value of peaty deposits during climafrost recession, change in character of peaty matter with depth, and relative disintegration value of the organic matter following disturbance and aeration.

In prescribing for such a varied list of requirements, the unit of structure had to be small enough to afford reasonable flexibility for reference, and to account for the main types of gross structure encountered. Also, the ultimate units could not be entirely arbitrarily chosen because it has been shown by analytical method (2) that organization of the terrain is revealed on a natural basis. Only that selection of entities which reflected these natural relations, therefore, would afford widest application and validity. This matter now requires further consideration.

Mere presentation of the 16 categories in Table I reveals little direct evidence of a system by which they have been derived. Examination of nearly 500 peaty samples secured primarily from an area of a 200-mile radius around Fort Churchill, Manitoba, shows that the ultimate peaty elements which predominate and which might be used appropriately to the specifications mentioned are few in number. Most prominent among them is a granular base composed of aggregates variable in size and shape. Because the shape of the aggregate (granules) is so very variable and consistently irregular, no designation other than "amorphous" seems applicable.

Besides the amorphous-granular, there is a fibrous element. Though there is only one type of amorphous-granular element (morphologically) there

are several types of fibrous elements. The fibres may be woody (derived from tissues originally lignified) or non-woody (originally non-lignified and probably cellulose in origin). They are either fine in texture (1 mm. or less in smallest cross-section) or coarse (greater than 1 mm. in cross-section), and this description holds for woody and non-woody types except for the fact that non-woody coarse-fibrous is extremely rare and for all practical purposes negligible. Size range in coarse-fibrous members may be considerable; the order of 1 cm. to 1 dm. is not infrequent.

Combinations of Macroscopic Structural Entities

There are cases where these primary macroscopic units of construction occur unmixed with others, but these are relatively rare and therefore their significance is uncertain. In one exception a mass of non-woody, fine-fibrous peat was buried several feet beneath the back-fill of a well-travelled highway (Provincial Highway no. 69 near Moonbeam, Ontario). Eventually the road bed subsided and high walls of non-woody fine-fibrous peat heaved up. The newly exposed peat formed flanking embankments to the reconstructed highway. However, had the highway route been established 50 feet to the north of the existing site in the first instance, embarrassment would have been avoided. The pure component was confined locally and was not so prevalent as the peat of mixed constitution with which it was surrounded and with which it is likely that subsidence would not have occurred.

All other categories are mixtures of two or more basic components, as an examination of Table I will reveal.

For the inexperienced field observer, it is sometimes difficult to decide the category to which a given example of peat belongs. This is usually for three reasons. One is that each category allows for secondary difference in macroscopic form within the category; arrangements of constituent particles may differ; size of macrofossils may vary depending upon the botanical origin of the particles; and relative sizes of constituents may differ to a degree. A second reason is that other physical attributes of the sample are variable, for example, water content or amount and form of incorporated ice. The third reason is that the observer has no set of standards (type examples) with which to compare the sample he is inspecting in the field.

Experiment has shown that, of the three, the last mentioned reason provides the greatest source of trouble and leads to indecision. Also, this is where the demand for assistance lies. To meet this difficulty, peat samples representative of the categories would be most useful as collateral reference material. Though the scheme is not entirely impractical, it is, for the time being, not as feasible as the provision of a set of photographs. To satisfy this need, Figures 1-16 are suggested. Reference is made to the primary structural entities in the description of the figures where this is deemed necessary.

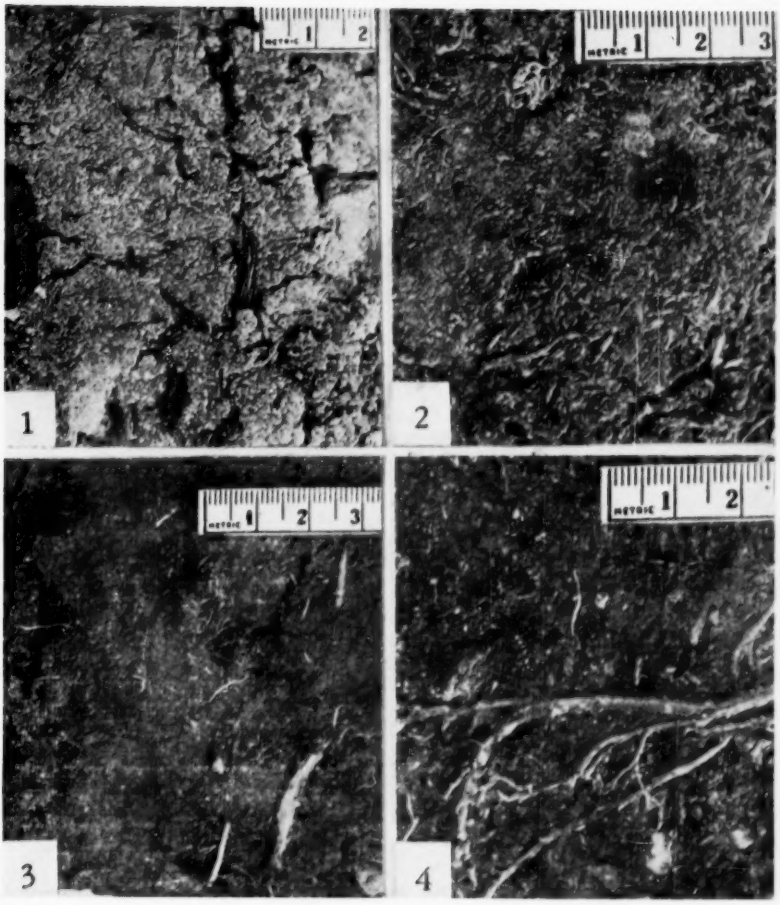

PLATE I. Sub-surface macroscopic construction of muskeg

FIGURE 1.—Category no. 1. Amorphous-granular.
FIGURE 2.—Category no. 2. Non-woody fine-fibrous.
FIGURE 3.—Category no. 3. Amorphous-granular in fine-fibrous.
FIGURE 4.—Category no. 4. Amorphous-granular in woody fine-fibrous.

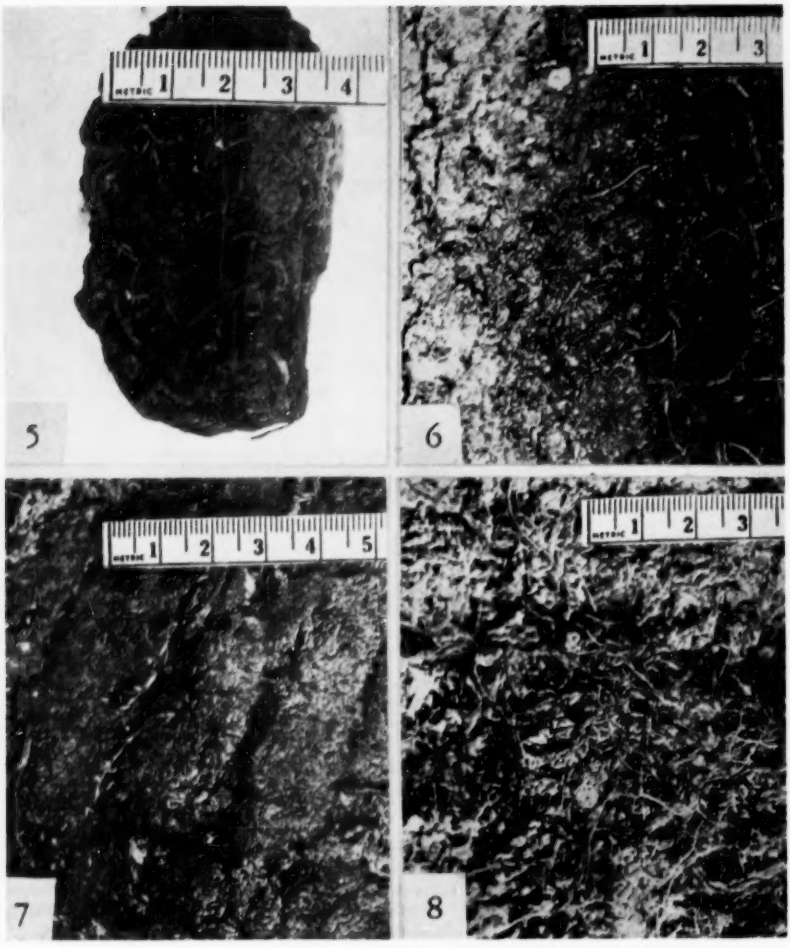

PLATE II. Sub-surface macroscopic construction of muskeg (*cont'd*)

FIGURE 5.—Category no. 5. Amorphous-granular, fine-fibrous, non-woody in woody fine-fibrous.

FIGURE 6.—Category no. 6. Amorphous-granular with woody fine-fibrous held in coarse-fibrous.

FIGURE 7.—Category no. 7. Non-woody fine-fibrous covering amorphous-granular in fine-fibrous.

FIGURE 8.—Category no. 8. Non-woody fine-fibrous; mound in coarse-fibrous.

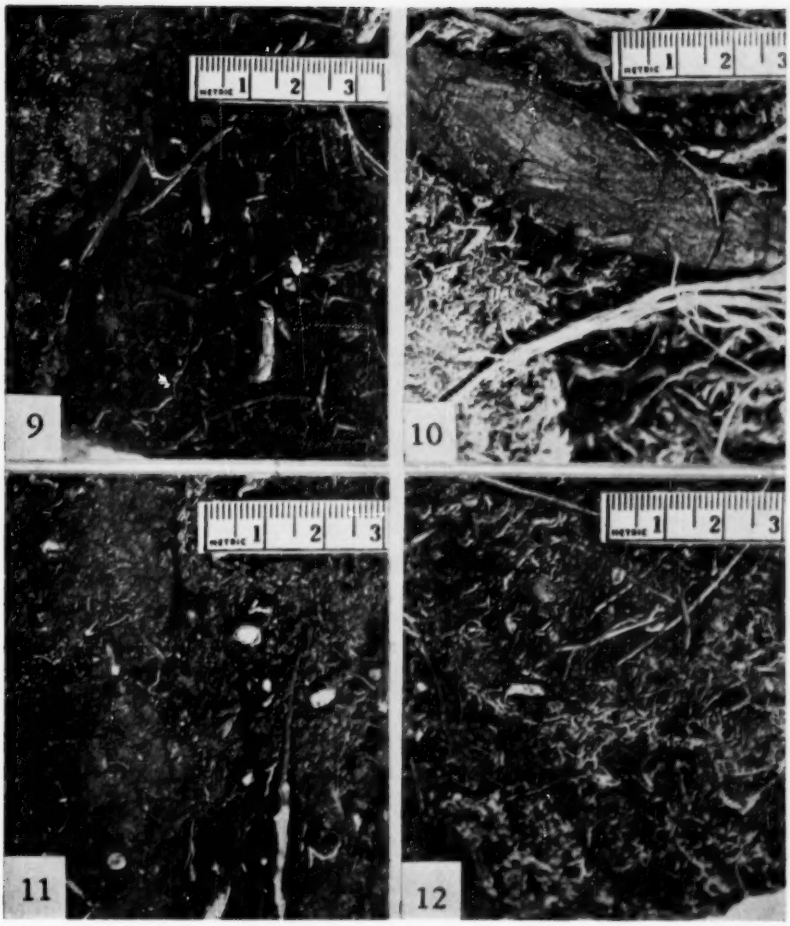

PLATE III. Sub-surface macroscopic construction of muskeg (*cont'd*)

FIGURE 9.—Category no. 9. Woody fine-fibrous held in coarse-fibrous.
FIGURE 10.—Category no. 10. Woody particles in non-woody fine-fibrous.
FIGURE 11.—Category no. 11. Woody and non-woody particles in fine-fibrous.
FIGURE 12.—Category no. 12. Coarse-fibrous, woody.

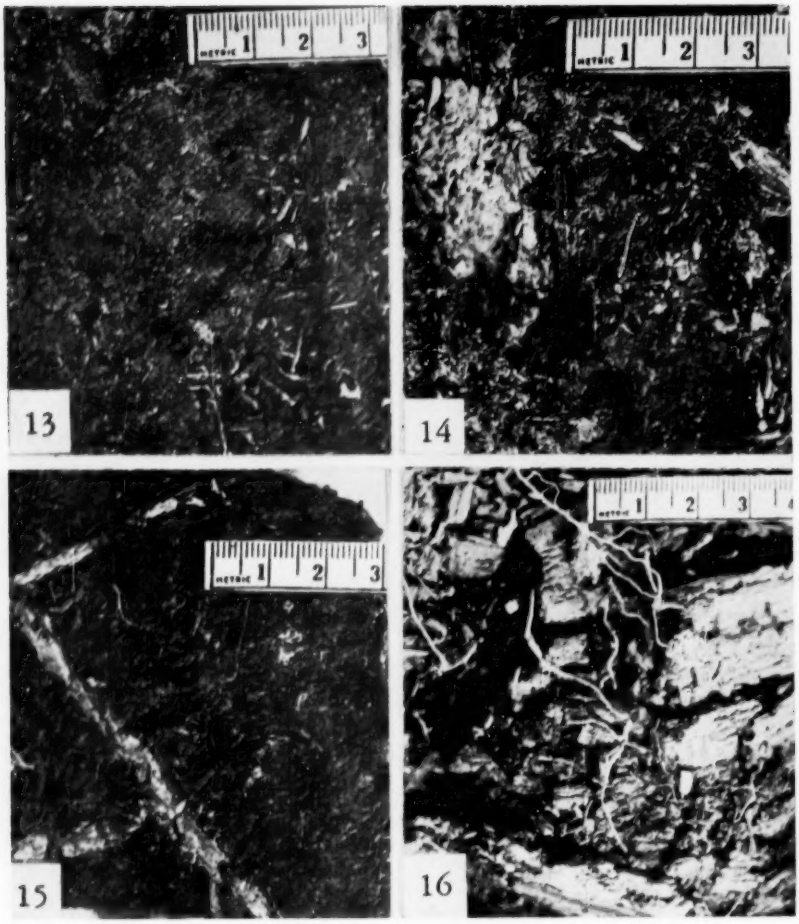

PLATE IV. Sub-surface macroscopic construction of muskeg (*cont'd*)

FIGURE 13.—Category no. 13. Coarse-fibrous traversing fine-fibrous.

FIGURE 14.—Category no. 14. Non-woody and woody fine-fibrous held in coarse-fibrous.

FIGURE 15.—Category no. 15. Woody with amorphous-granular in fine-fibrous.

FIGURE 16.—Category no. 16. Woody coarse-fibrous with scattered woody erratics.

Biological Justification of Structural Categories

That a relationship exists between micro- and macrofossils in given examples of peat was suggested in an earlier work (3). This provided limited evidence supporting a view that the occurrence of macrofossils, like the historical sequence of microfossils, reflected a natural organization in the peat matrix.

The use of categories (combinations of primary elements) has introduced another problem concerning the validity of the system of reference as a whole. Combinations of units are not necessarily the equivalent of single primary elements, and physical association may not justify assumption of biological association. Though the latter suggests a natural condition, the former may be to a degree, or even completely, artificial. The same contention might apply with respect to the condition governing the selection of categories. Also, the selection of categories was based, to some extent, on occurrence. There may be no claim for biological correlation here.

If the degree of artificiality involved should prove to be high, this would interfere with valid assessment of peat properties which must, by requirement, reflect natural organization. The measure of dependability of the categories as reference factors is therefore related to microfossil sequence. To be highly dependable, biological comparisons must be manifest.

Structure in Depth

There are very few 6-inch square borings which do not show some structural variation. This can be readily seen on the longitudinal faces of the sample when it is taken from a selected site in the organic terrain. Character variation was clearly expressed on the basis of microfossil sequence by the writer elsewhere (3). In the same account it was held that relationship existed between microfossil sequence and macroscopic content. However, reference was made to the latter in terms of primary structural elements; presence of structural categories (Table I) had not been adequately revealed or assessed at that time.

Where observations on macro-structure were given, the predominating primary element was named to identify the example as a whole. Thus for one type of microfossil sequence of relatively similar constitution throughout depth, it was indicated (2, p. 12) "that the macroscopic or visible component of the peat that will predominate is of the fibrous ribbon-like structure, and that this condition will hold for the entire depth of the muskeg at that place." On the other hand, in an example for which the pattern of frequency in microfossil sequence changed with depth, it was suggested that (2, p. 12) "a gross structure predominantly non-fibrous and non-woody changing near the surface, however, to a slightly fibrous-woody constitution," would obtain.

It would seem that, if the use of categories (Table I) is to be suitable and reliable for all cases, it should be applied to that type of gross sample potentially high in structural variation. This would afford, for the most

problematical type of organic terrain, assessment of the system of identification of macro-structure. Accordingly, by prediction from surface observations (2, p. 12), a gross sample of peat most likely to exhibit variation in macro-structure with depth was chosen for analysis from the organic terrain at Chesnaye near Fort Churchill, Manitoba. Apart from the attempt to select for maximum variation, the sample was chosen without thought of possible relationship with oecological phenomena.

This sample, about 8 inches square in cross-section, was 40 inches in depth and represented the total thickness of the organic layer at the site. Another preliminary observation, which might have been made by anyone not familiar with the categories for identification of macro-structure, revealed that there were zones of different thickness recognized as layers at consecutive levels in the column. The physical or chemical variants which made it possible to detect these strata were colour, particle arrangement, to some extent density of material, and other features, elusive, ill-defined, or impracticable as reference designants.

Microfossil analysis of the sample then followed, and the microfossil histogram patterns for the entire depth are shown in the eight columns of Fig. 18. The procedure for this has been described by the writer (2, p. 8). For the present analysis, a slight departure was allowed to facilitate comparison of micro- and macro-structure; the eight columns correspond to the selected strata of different magnitude, not to equal intervals of depth. The plant equivalent of "index unit" is explained in (3, p. 63). However, the writer wishes to emphasize features of frequency trend rather than plant succession and, as is held in (3), the adoption of "index unit" avoids risk of prejudice on a botanical basis.

Examination of the histograms in Figure 18 shows a shift in pattern that is not erratic but orderly and thus reflects organization sustained but changing. The trend towards shift is largely due to lessening prominence (but not disappearance) of index unit IX from right to left (base to top of peat) and somewhat of a rise to prominence of units V and VII. To a lesser extent, frequency-increase in the same direction is noted for units IV and VIII. It will also be noted that at the 15.5 inch depth, the trend is suddenly emphasized. Perhaps with unit III as an exception, the first three index units show no marked frequency trend. At the 15.5 inch depth, unit X overtakes IX and the former on the average exceeds the latter in prominence for the upper half of the peat.

Application to this example of the identification system for macro-structure (Table I) was also achieved. From the base of the column towards the top for a distance of 2 inches, category 3 applied—amorphous-granular in fine-fibrous (cf. Table I; Fig. 3). For the next 14 inches, category 2 occurred—non-woody fine-fibrous (cf. Table I; Fig. 2). The next 15 inches were identified as category 11—woody and non-woody particles in fine-fibrous (cf. Table I; Fig. 11). The final 9-inch segment to the top of the

peat was designated as category 13—coarse-fibrous traversing fine-fibrous (cf. Table I; Fig. 13).

No difficulty was encountered in identification; the four categories mentioned were clearly marked. The fact that the zone limits of the categories did not coincide with those of the eight strata that had been acknowledged and used as limits for analytical purposes requires some consideration. It can be demonstrated that demarcation in peats, though due to physical or chemical factors, is not always strictly structural (cf. p. 60). This was the case with regard to the eight strata in question. Attempts to rationalize these zones for identification purposes for general application would lead to taxonomic inconsistency. The macro-structure category system, however, which relies only on structure and presence or absence of woody remains, reflects the true nature of the constituent primary entities whatever the conditions surrounding the existence or distribution of the peat, and is thus taxonomically dependable.

Choice of taxonomic procedure, particularly in relation to natural requirements, can better be assessed if the macro-structure system is compared with a classification system already known to be dependent on natural attributes. The appropriate choice here is obviously the microfossil system of designation, which portrays the developmental history of the peat. For the test case analysed in this work, all but the top one-quarter of the peat was essentially characterized by the fine-fibrous primary element. Thus, the named macro-structure of the peat is correlated with its microfossil index equivalents (index units IX and X, principally IX) which are identified as to natural attributes through direct generic connection. Also, the introduction of woody and non-woody erratics in the top half of the lower three-quarters of the peat is coincident with a rise to prominence of index units V and VII and VIII. These are undoubtedly equivalents of the erratics and the true botanical clue to the origin of the coarse-fibrous primary element which arises significantly in the top quarter of the peat. They also reflect natural relationships on the basis of the generic implication.

The comparisons reveal two other observations for which the writer claims significance. The identification of macro-structure contributes little or nothing when an attempt is made to reconcile the high incidence of index units I, II, and III in the microfossil analysis (Fig. 17). Secondly, the macro-structure system does not record change in generic source of the fine-fibrous primary element manifest in the microfossil record at the 15-inch level.

It has been demonstrated elsewhere (3) that indices I, II, and III, which represent *Pinus* sp., *Picea glauca* (Moench) Voss, and *Picea mariana* (Mill) BSP, have least influence in peat development. These and other arboreous types, though important as time and climate markers, are not so significant as shrub and herbaceous pollens and spores, which to a greater extent have originated *in situ* and therefore are more significant as indices of agents

incorporated within the peat in the course of its development. For this reason, the fact that the macro-structure system apparently partly eliminates microfossil indices I, II, and III does not suggest artificiality. It avoids artificiality and points to the acknowledged limitation in the microfossil system.

The failure of the macro-structure system to reveal change in origin of non-woody fine-fibrous content can be claimed to reflect some artificiality. That this is serious is questionable because, with the advent of *Betula* sp. and Ericads, the plants responsible for index units V and VII respectively, the switch in prominence from IX to X is a natural feature in the synoecological sense. This is substantiated by field observation and by laboratory record.

It now seems reasonable to conclude that the design of the macro-structure system of identification and classification is allied to biological trends and natural adjustments in the development of organic terrain. Therefore the feasibility of estimating relationship of structural change to depth of organic terrain on a rational and consistent basis seems to be established.

If this conclusion is justified, it would be safe to predict that the occurrence of categories of macro-structure would be repeated because the biological phenomenon with which the category or categories would be associated would not likely be restricted to one site, but would be widespread and controlled by microclimatic, edaphic, topographic, and biotic factors. Also, for the same reasons, it might be expected that some categories and sequences of categories would be more prevalent than others.

Relative to these points, only preliminary studies on range of structural variation have been attempted. Though the results are inconclusive, they do lend themselves to the establishment of principle and provide instructive collateral evidence for that already presented for the type example studied intensively.

To provide the information, 87 gross peat samples were identified according to the major macro-structure category representative throughout. In the majority of cases, for applied studies, this is apparently adequate and in actual fact, many, if not most examples would show that not more than a single category-reference is necessary to designate the example. On this basis, several categories occurred very frequently. Seven (numbers 2, 3, 4, 6, 7, 11, and 15) occurred more than half a dozen times. Number 4 occurred eleven times, number 2, twelve times, and number 3, showing highest frequency, seventeen times. Only one category, 16, did not occur among the examples examined. This is because it is difficult to secure a representative sample; the large woody chunks make sampling difficult.

After such confirmation, though on a limited scale, that macro-structure in depth is repeated throughout terrain and that some categories are commoner than others, it is now appropriate to examine macro-structure in depth with respect to geographical aspects. Fig. 17 shows the source-distribution for seventy-four gross peat samples in the vicinity of Fort Churchill,

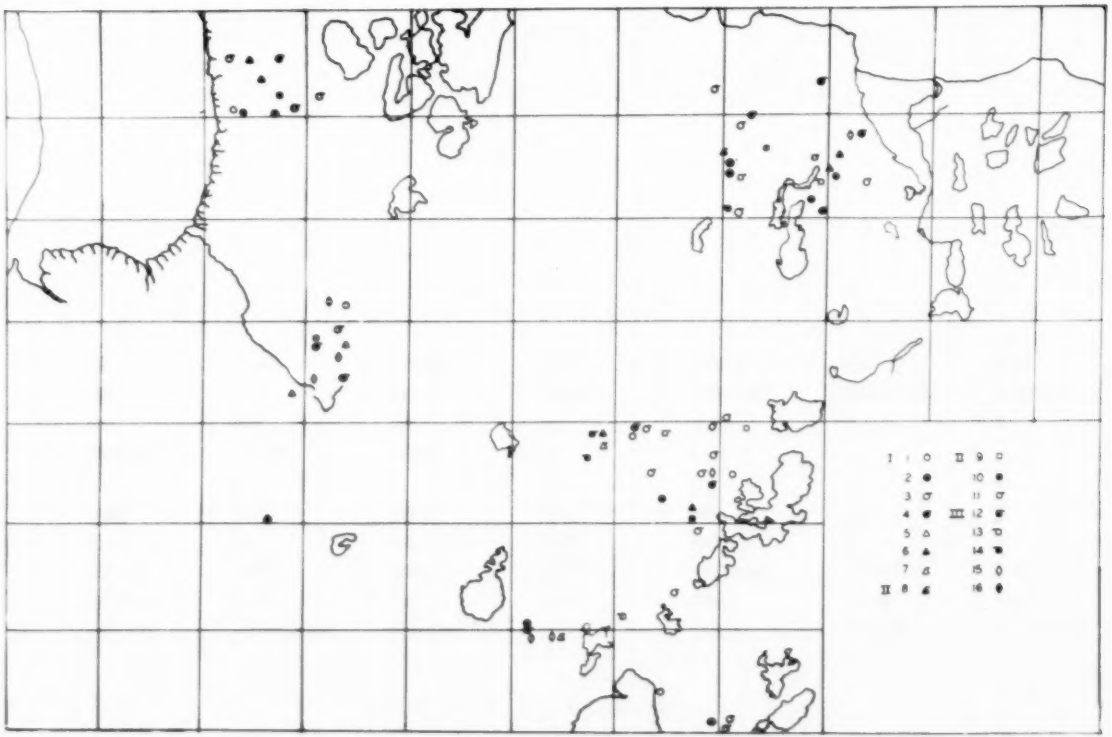

FIGURE 17.—Outline map of district immediately south of Churchill, Manitoba, with symbols indicating macrostructural constituents of muskeg samples.

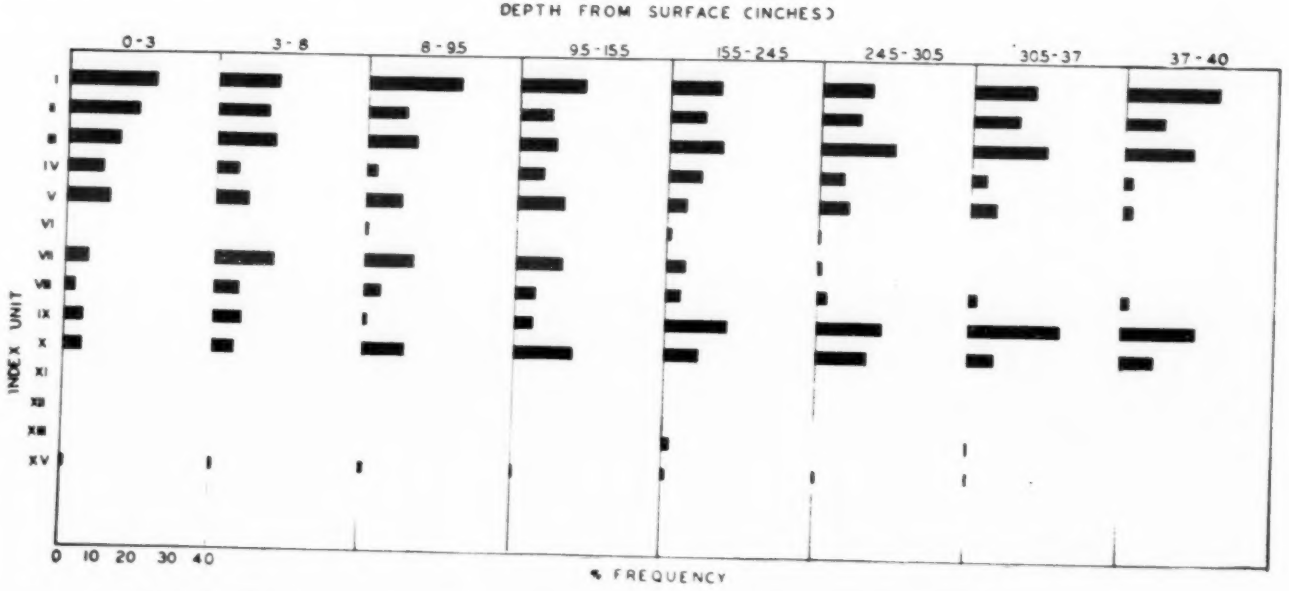

FIGURE 18.—Histogram patterns from 40-inch bore showing sequence of index unit frequency in depth.

Manitoba. Each example is identified according to macro-structure category (cf. Fig. 18 and Table I). Examination of the distribution suggests that for all four major groupings non-woody fine-fibrous matrix was common. In the area with most examples this is substantiated by category 3 (amorphous-granular in fine-fibrous, Fig. 3) which represents over 30 per cent of the sites examined. For another area, Fig. 17 upper right, which has fewer examples, the absolute number of sites showing a woody fine-fibrous primary element (in category 4) is twice as great as for the first area. This, along with other evidence of coarse-fibrous woody constitution which summarizes the situation for categories 6, 10, 11, 13, 14, and 15, suggests that, on the whole, the organic terrain is more generally woody and of coarse, resistant texture than is the case for the former area. Examination of a third area (Fig. 17, upper left) suggests that woodiness and coarse texture is perhaps more sporadic than for the former case. Finally, a fourth area (Fig. 17, lower left) by reason of the relatively high tendency towards woodiness, seems to be almost completely characterized by very coarse peats with a woody foundation.

There is a question as to whether in the last two areas an insufficient number of examples has been secured to warrant the decisions made. This would probably be so if it were to be claimed that a statistical analysis has been made. The latter will provide a basis for investigation in the future. The present work, it is thought, suggests validity of comparison on a qualitative basis only. Acceptance of this for the third and fourth mentioned areas is perhaps strengthened when it is considered that initial field investigation, through the application of random testing for structural variation, suggested that the number of samples was sufficient to justify empirical results required.

Bearing Capacity Assessment

Resistance to compressional force by organic terrain has been difficult to assess mainly because of lack of evidence to demonstrate organization in peats on a macro-structural basis. Also, difficulties in this connection have arisen because there is lack of knowledge concerning the structure of the mineralized basal component of organic terrain.

Relative to the first of these limitations, it is hoped that the information resulting from this investigation will be of some help. The macro-structure categories provide what is thought to be adequate facilities for identification in terms of qualities that are directly related to bearing potential. Their application can be expected to reveal and prescribe for orderly association of structural facts, with bearing potential data likewise appropriately aligned. In that the categories closely conform to conditions that depend on natural attributes, as demonstrated in this account, trend in structural-variation range and change in macroscopic structure, whether slight or great as expressed by category description, apparently reflect biological organization and the circumstances controlling oecological phenomena

characteristic of peats. Again, because of the inevitable connection between bearing potential and macro-structure category, it may be expected that bearing potential characteristics and conditions can be intelligently and fully interpreted in all their aspects and implications.

The inference that may be drawn from the evidence in Figure 17 relative to bearing potential is perhaps trivial unless it is claimed that the information has assisted in the establishment of the principle that variation in bearing potential of organic terrain can be portrayed through the macro-structure categories. Resistance to compressional force will increase directly for the four areas shown in Fig. 17 in the order discussed (p. 65). With additional data, some of which may now be safely predicted from collateral information, bearing potential over selected subsidiary areas or linear configurations within any of the four areas can now be devised.

The limitation on estimating bearing potential mentioned above (p. 65) as a possible function of the constitution of the mineral foundation layer associated with the peat, is still to be examined. Its effect will doubtless show in quantitative computation for given depths of organic terrain. It can only be claimed here that some evidence is at hand which demonstrates that grain size of particles in the mineral component tends to increase as woodiness and coarseness in the peaty component above the mineral become more prevalent.

With the macro-structure categories correlated with mineral foundation layer, and the former now prominent as the basis of reference for the structural pattern of terrain, the approach to interpretation of drainage relations now becomes clearer and with it an even better understanding of bearing values is possible. The final word on a complete interpretation of bearing potential cannot be given, however, until implications introduced by vegetal coverage and topographic variation are understood. This may be gained by consulting other accounts (2, 3, 4).

Acknowledgments

The writer is grateful for the continued support of the Associate Committee on Soil and Snow Mechanics, National Research Council of Canada and the Arctic Research Section, Defence Research Board; the former assisting work on muskeg ground studies and the latter contributing towards organic terrain studies from the air.

To Robert F. Legget, Chairman of the Associate Committee on Soil and Snow Mechanics, the writer's indebtedness increases. Miss Jean Evel has given invaluable assistance. Her initiative, with that of Mr. W. J. Thorne, another who has contributed directly in the work, has done much to facilitate the organization of data. Messrs. R. Eydt and P. Trip have lately assisted and their efforts along with those of many earlier associates who provided data from the field, are greatly appreciated by the writer.

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SECTION FIVE

Assessment of Early Differentiation in *Pinus* Proembryos Transplanted to *in vitro* Conditions

NORMAN W. RADFORTH and LOUISE C. PEGORARO

Presented by GLEB KROTKOV, F.R.S.C.

Introduction

ENLIGHTENMENT regarding the phenomenon of growth and the problems involved in its regulation is now being widely pursued. In the past, the main part of this study was confined to whole organisms or cells of differentiated tissues and organs. Recently, attention has been focused on embryonic plant material. This supplies cells which contain the potentialities for extensive growth and differentiation but which, in that they do not reveal the expression of these potentialities, are not apparently limited by obscure pre-existing growth determinants.

Because of this, embryonic material would be desirable for the study of some of the fundamental problems of growth. It would be particularly useful to determine the extent of phylogenetic control on the early development of plants, for, if it is the major factor in the expression of growth, such material would not have as wide appeal in growth studies.

Plant embryos are difficult to utilize for *in vitro* studies, owing to their minuteness and close association with parental tissues. These handicaps make technical procedures very difficult. It is virtually impossible to dissect and process such small groups of cells, using ordinary methods.

The difficulty could be circumvented if conditions similar to complete excision could be obtained by development of new technique. This would involve the removal of excess parental tissue and nullification of the remainder, making it relatively ineffective in influencing the growth of the embryonic cells. The close association with the parental tissue presents a further problem as, in most cases, this tissue is the source of nutriment for the embryo. Thus the question arises whether the embryos, especially young ones, will survive and grow in an artificial medium.

Van Overbeek *et al.* (16 and 17) have succeeded in growing young *Datura* embryos *in vitro*. Still smaller embryonic masses successfully cultured were the proembryos of *Ginkgo*, by Radforth (10), and of *Pinus*, by Woods (15).

These latter types of embryonic masses, the proembryos, are very small groups of cells with the minimum degree of differentiation. Both Radforth and Woods found that the development of these masses was not entirely

controlled by phylogenetic factors but that physiological factors were influential. However, there are still obstacles preventing the use of this material. One of the more important is the lack of an adequate basic medium which will support optimum growth *in vitro*. The medium used by Woods (15) for *Pinus* was not thought to be a satisfactory one for *Pinus*.

In spite of these difficulties, it was hoped that successful culture *in vitro* of the proembryo of *Pinus nigra* var. *austriaca* could be accomplished. This work was designed to test the hypothesis of Radforth (10) regarding the importance of suspensorial development in phylogeny, to evaluate any changes in the growth expression accompanying a change in environment, and to contribute information regarding the basic medium.

Methods

To facilitate careful and accurate dissection, the micromanipulator (3) was used. The female sporophyll was cut along a median line, from the distal to the proximal end, to afford ready removal of the scales. Using a sterile probe, the prothallium was removed from its integument and placed in a covered, sterile, moist chamber of the micromanipulator. This chamber was provided with a floor of sterile 4 per cent agar gel, to prevent excessive breakage of the glass implements of the micromanipulator. The chamber was then placed on the stage of a dissecting microscope and the bulk of the gametophytic tissue was removed, leaving only a layer, several cells in thickness, surrounding the embryonic masses. This layer was left to provide ease in manipulation but was designed to permit the inclosed proembryos to have a nearly uniform environment in the culture medium.

In order to ensure that all stages from fertilization to the establishment of the proembryo would be obtained, the dissections were begun on June 23, 1954, one week before the time claimed for fertilization (2) and continued on every second day until July 19, 1954.

During the whole procedure, precautions were taken to provide and maintain as high a degree of sterility as possible. Besides autoclaving media and agar and dry sterilizing (heat for one hour at 170°C) the glassware involved in the transfer of nutrient medium, the surrounding work area and implements were made sterile. This was accomplished using three fluids; 70 per cent ethanol, 5 per cent aqueous phenol, and calcium hypochlorite solution, prepared by adding 20 g. of calcium hypochlorite to 280 cc. of water and filtering after allowing it to stand for 3 hours. Each has disadvantages which prevent its use throughout the whole procedure. The 70 per cent ethanol was used to sterilize the glass implements and moist chamber of the micromanipulator; the phenol for the working area, the equipment, and surrounding air; the calcium hypochlorite was used to cool the flamed metal instruments and to decontaminate the inocula. Calcium hypochlorite solution does not affect plant cells if used for a short period of time, but is effective against surface contamination (10).

The dissected material was removed from the chamber, using a small,

sterile wire loop, and placed in the calcium hypochlorite solution for ½ to 1 minute, and then transferred to a tube containing a medium. The mouth of the tube was flamed before and after inoculation.

The source of mineral salts for the medium was modified Crone's solution. This was the one used by Radforth (10) for *Ginkgo*, and preferred by Woods (15) to Knudson's formula for *Pinus*. It is prepared as follows: 10 g. potassium chloride; 2.5 g. calcium sulphate; 2.5 g. magnesium sulphate; 2.5 g. magnesium phosphate; 2.5 g. iron phosphate are thoroughly mixed and one litre of distilled water is added to 1.5 g. of this mixture. This is referred to as "100 per cent" modified Crone's solution. By adding distilled water to this solution in the correct proportions, 50 per cent, 25 per cent and 12.5 per cent concentrations of modified Crone's solution were obtained. The hydrogen-ion concentration was adjusted to a pH of 5.5.

Following this, each solution was divided into eight portions, and to each, one of the following nutrient combinations was added: dextrose, 2 g. per 100 cc.; yeast extract, 2 g. per 100 cc.; dextrose, 1 g. per 100 cc. plus yeast extract, 1 g. per 100 cc.; dextrose, 1 g. per 100 cc.; yeast extract, 1 g. per 100 cc.; sucrose, 1 g. per 100 cc.; sucrose, 1 g. per 100 cc. plus yeast extract, 1 g. per 100 cc.; sucrose, 2 g. per 100 cc.

The resulting twenty-four solutions were dispensed in 10 cc. lots in ordinary 20 cc. test tubes, which were then fitted with cotton plugs and autoclaved for 20 minutes at 15 pounds' pressure and 100° C.

To investigate its usefulness in preventing contamination in this work, penicillin was dissolved in sterile, distilled water and added to half the media, so that each ml. of the resulting solutions contained 10 units of penicillin. The media were dispensed, under sterile conditions, into sterile serological tubes; each tube received 0.5 ml. of medium. These tubes were also fitted with cotton plugs.

The inoculated media were placed for 10 days in an insulated cabinet where the temperature fluctuated between 25° C. and 28° C. The incubation period was chosen arbitrarily on the basis of Woods' (15) findings. The fluctuation was not considered to be injurious to the growth of the tissues as it was found that there was a variation in the internal temperature of the cone in its natural state and this variation exceeded that in the cabinet. To determine this internal temperature, a thermometer was inserted into the cone while on the tree. Temperatures were recorded for several cones which differed in size, relative position on the tree, degree of sunlight, and external temperature.

After the incubation period, the tubes were removed from the cabinet, their media decanted and replaced with distilled water as a wash. This in turn was decanted to be replaced by Carnoy's solution (6), for killing and fixation. This was allowed to remain for one-half an hour. The pieces of tissue were then removed and placed in vials, containing 70 per cent ethanol; the vials were corked and stored in a refrigerator to prevent excessive evaporation.

After the storage period, the tissues were transferred to the various solutions of the normal butyl-ethyl alcohol series (6) for dehydration and infiltration. The material was embedded in paraffin wax (Tissuemat) and sectioned to a thickness of 12 microns, and the sections were mounted on slides using Meyer's adhesive (6).

The Tissuemat was removed and replaced with water using the method outlined by Johansen (6). Following this, the sections were submitted to a period of hydrolysis in 1 N. HCl at 60° C. for 6 minutes. The hydrolytic medium was as recommended by Hillary (4) and the time was determined experimentally for this particular material by using different periods for hydrolysis. The period which produced material giving the most intense reaction with Feulgen's reagent was selected for this experiment. After rinsing with distilled water, the sections were treated with the Feulgen reagent, prepared according to Hillary (4), for not less than 4 hours. This period of reaction was also arrived at through experimentation.

Removed from the reagent, the sections were placed in three differentiated baths of sulphur dioxide water, each 10 minutes in duration; washed in running water for 5 minutes; dehydrated using the ethanol series; cleared in clove oil solution (6); treated with xylol and mounted in Canada Balsam.

In vivo material, to serve as controls, was excised on each day of excision and for 10 days after the last excision date. The controls were placed immediately into Carnoy's solution and underwent the same procedure outlined for the *in vitro* material.

Results

Examination of the *in vitro* material indicated that proembryonic growth had occurred in 37.3 per cent of the 672 cultures (plus 90 controls). Growth was assessed on the basis of one, or a combination of the following criteria:

1. The appearance of mitotic figures in the *in vitro* material.
2. The increase in mean cell size as compared to the cells of the *in vivo* material.
3. The increase of tissue mass, relative to *in vivo* conditions.
4. Change in growth direction.
5. The appearance of developmental forms not encountered *in vivo*.

Media with yeast extract and sugars added supported the highest percentage of growth and provided the most consistent results. The percentage of cases indicating growth in dextrose and yeast extract was 57.1 per cent and in sucrose and yeast extract it was 55.5 per cent. Those media with only sugars added were not as efficient in supporting growth, nor were they as consistent. When 1 per cent of sugar was added, the decrease in this ability was greater than when 2 per cent of sugar was used. There was very little difference between the two sugars, dextrose and sucrose, in their ability to support growth, except at the 2 per cent level. Tissues grown in the medium with sucrose added in concentration of 2 g. per 100 cc. displayed growth similar to that which occurred in tissues grown in media with both sugar

and yeast extract added, rather than to that displayed in material grown in media with dextrose added. The evaluation of the differences or similarities in growth is based on the accumulation of information concerning the position and condition of healthy and declining cells. This was derived from the inspection of a large number of slides. The media with only yeast extract added supported only a very low degree of growth. The incidence of growth in the medium with 2 per cent yeast extract added was 22.0 per cent, and in that with 1 per cent added was 28.6 per cent. However, this growth was not extensive.

The incidence of growth was higher in media with the higher concentration of modified Crone's solution. The difference was greater between the 25 per cent and 12.5 per cent concentrations than between the 50 per cent and 25 per cent concentrations.

Contamination occurred in only 7.8 per cent of the 672 cultures. This was considerably lower than that experienced by Woods (15), who found contamination to be a serious inhibitor. Penicillin, in the concentration used, was found to be ineffective in preventing contamination, occurrence of this being slightly highest in the penicillin-containing media. However, this difference was not significant (8.0 per cent contamination in the penicillin-treated media as compared to 7.7 per cent in the non-treated media).

Great irregularity with respect to the date of fertilization in this species was noted. This is in accordance with the findings of Woods (15) but in disagreement with Coulter and Chamberlain (2), who set the fairly rigid date, July 1, for the fertilization of *Pinus*. Corroborating the observations of Woods still further, it was noted that there was a variation, with respect to fertilization, among the archegonia of a single prothallium, and among the prothallia of a single sporophyll. Fertilization occurred most frequently between June 27 and July 1, but it was also observed as early as June 25 and as late as July 5.

Examination of the *in vitro* embryonic masses indicated that there were several variations in growth as compared with the *in vivo* material.

The conditions of the healthy embryonic cells *in vitro* were much similar to those occurring *in vivo*, except for some of the smaller groups found in the chamber. In these the nuclei were smaller and the cytoplasm was not so dense (cf. Figs. 2, 12). In some masses there were various degrees of plasmolysis and nuclear decline, most frequently in the tissues cultured in the poorer media. In some proembryos, cultured in media with only yeast extract added, the response to the Feulgen nuclear reagent indicated that desoxyribose nucleic acid had diffused into the cytoplasm.

There was a significant increase in the number of groups of embryonic cells *in vitro*, especially in the later stages of embryonic development. For example, seven groups were seen in one piece of tissue, while *in vivo* material of comparable embryonic development would have about three proembryos. This increase in the number of groups of embryonic cells, as compared to *in vivo* state, was found in all material in which growth had taken place.

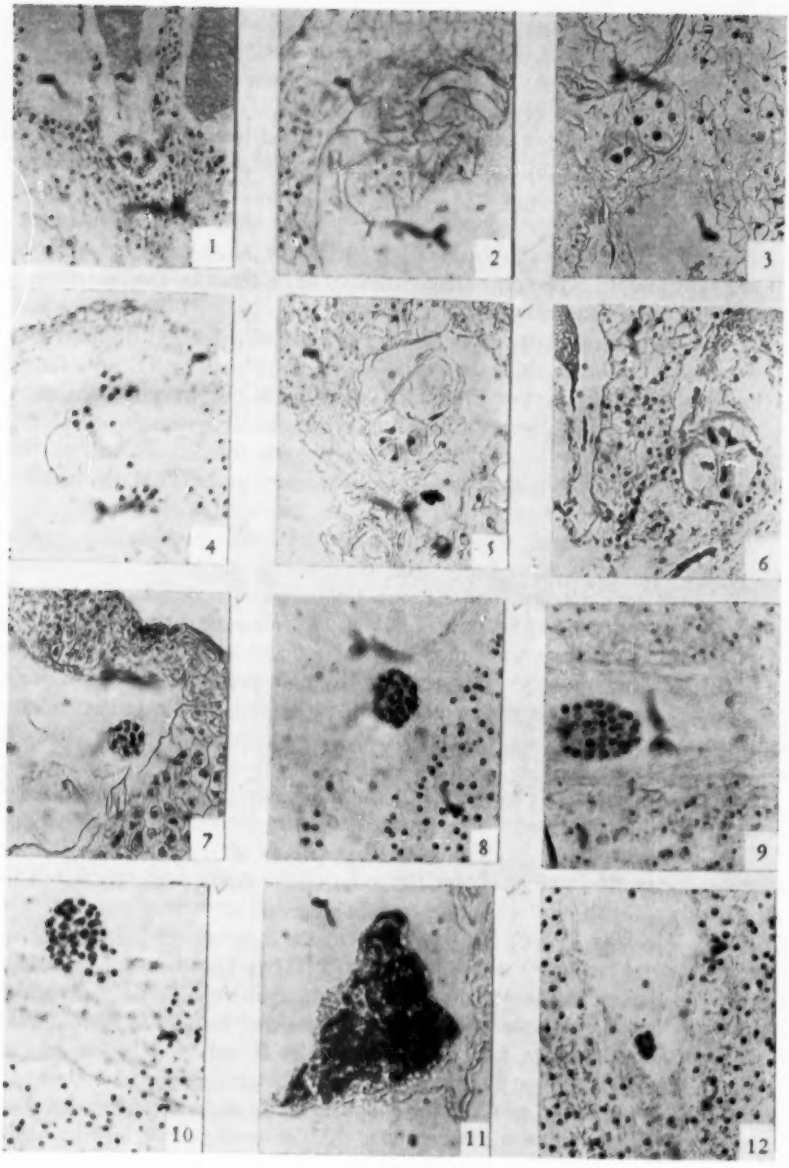

PLATE I

In vitro the frequent occurrence of small- or medium-sized groups was observed, while *in vivo* they were seldom encountered except in the stages of suspensorial elongation and proembryo establishment. The number of the larger groups of embryonic cells that were seen *in vitro* was closely related to the number of proembryos seen in the *in vivo* material of comparable embryonic development. In almost every piece of material examined in which growth had taken place, a range in the size of the embryonic masses was exhibited. The size of the group of embryonic cells was measured by the number of nuclei seen in the largest section of the group rather than by the physical extent of the mass. The groups with fewer than 8 nuclei were classified as small; those with 8 to 20, as medium; and those with more than 20 nuclei as large (cf. Figs. 3, 7, 9).

These groups occurred in various positions within the prothallial remnants, and two terms are used to assist in the description. The term "channel" refers to the cavity created in the prothallium at the time of suspensorial elongation. This channel increases in size as embryonic development progresses and is conical in shape with its base opening into the chamber. The "chamber" is the space previously occupied by the archegonial cluster. Embryonic groups were seen throughout the channel and chamber, and generally there was a correlation between the size of the groups and their posi-

PLATE I

FIGURE 1.—Cells of two-tiered stage showing weak radial arrangement. Mag. 85X. In culture June 29–July 9. Medium: 50% mod. Crone's solution plus 2% yeast extract.

FIGURE 2.—Loose, radial arrangement of embryonic cells in chamber. Mag. 85X. In culture July 17–27. Medium: 12.5% mod. Crone's solution plus 2% dextrose.

FIGURE 3.—Embryonic cells located in chamber. Mag. 85X. In culture July 11–21. Medium: 12.5% mod. Crone's solution plus 1% sucrose.

FIGURE 4.—Several small groups of embryonic cells located in chamber. Mag. 85X. In culture July 15–25. Medium: 25% mod. Crone's solution plus 1% sucrose and 1% yeast extract.

FIGURE 5.—Group of embryonic cells located in chamber. Mag. 85X. In culture July 11–21. Medium: 25% mod. Crone's solution plus 1% sucrose and 1% yeast extract.

FIGURE 6.—Group of embryonic cells enclosed in jacket. Mag. 85X. In culture July 5–15. Medium: 50% mod. Crone's solution plus 1% sucrose and 1% yeast extract.

FIGURE 7.—Radial embryonic mass located high in chamber. Mag. 85X. In culture July 15–25. Medium: 12.5% mod. Crone's solution plus 1% sucrose and 1% yeast extract.

FIGURE 8.—Radial embryonic mass located in channel mouth. Mag. 85X. In culture July 15–25. Medium: 12.5% mod. Crone's solution plus 2% dextrose.

FIGURE 9.—Large embryonic mass in lower region of channel showing radiality. Mag. 85X. In culture July 13–23. Medium: 25% mod. Crone's solution plus 1% dextrose and 1% yeast extract.

FIGURE 10.—Mass in channel mouth showing reversed polarity. Mag. 85X. In culture July 7–15. Medium: 12.5% mod. Crone's solution plus 2% dextrose.

FIGURE 11.—Mass located in chamber showing disorganized nuclear material. Mag. 330X. In culture July 13–23. Medium: 25% mod. Crone's solution plus 2% sucrose.

FIGURE 12.—Proembryo in lower region of channel. Mag. 85X. *In vivo* material excised July 11.

tion. The largest groups of cells were most frequently found in the lower third of the channel and the size decreased as the distance from the chamber decreased. However, there were exceptions to this, as in several instances relatively large- to medium-sized groups were observed in the chamber or only a short distance from it, in the channel. In the lower regions of the channel several small- or medium-sized groups were seen, but these were closely associated with the edges of the channel. There did not appear to have been any preference in position in the chamber as embryonic groups were seen throughout this region (cf. Fig. 4).

According to the literature (2, 5, 9) and to observational experience, the proembryos *in vivo* are oblong in shape with parallel sides and rounded tips (cf. Fig. 12). However, *in vitro* the proembryos exhibit radial or three dimensional growth to a greater or lesser extent. The degree of this radiality was generally found to be least in the larger groups, and increased as the size of the mass decreased (cf. Figs. 7, 8, 9, 10). However, several large masses were observed which showed pronounced radiality.

Because radiality indicated some relation to size, and size to position, there was an apparent relationship between radiality and position. Generally, the masses tending towards maximum radiality were found in the chamber and the degree of radiality decreased as the distance from the chamber increased (cf. Figs. 7, 9). In the lower regions of the channel, however, there were large masses that exhibited pronounced radiality. In addition, some of the large groups located in the chamber indicated definite radial tendencies and, conversely, the small groups of embryonic cells associated with the channel edges indicated a very low tendency towards radiality.

From the literature (2, 5, 9) and from inspection of the *in vivo* material it was found that the axes of the proembryos coincided with those of the archegonia. *In vitro*, when radiality was not maximum, this was not observed in every instance; in some proembryos the axis of development was seen at various angles to the axes of the archegonia. The extreme expression of this tendency was revealed in those groups where the direction of growth was completely opposite to that *in vivo* (cf. Fig. 10).

The phenomena of increase in numbers, variation in the size of groups, the variation in growth direction and radiality appear to be related to the medium in which the tissues were grown. Material cultured in media with both sugar and yeast extract added showed the greatest number of embryonic groups which were larger and exhibited a greater degree of radiality than those cultured in other media. Also, in these media, there was an increase in the appearance of groups with abnormal directions of growth and in the number of exceptions to the generalizations drawn with respect to size, position, and radiality.

There was a decrease in the incidence and extent of these alterations in the expression of embryonic growth when only carbohydrate was added to

the modified Crone's solution. This decrease was greatest when 1 rather than 2 per cent carbohydrate was added.

There were a few abnormal phenomena which occurred in only one or two instances, but which are worthy of note at this time. One, as shown in Figure 6, is the appearance of a group of embryonic cells located within the archegonium, completely enclosed by jacket cells which showed little if any distortion of shape as compared to that for the very early post-fertilization period. The jacket cells also did not show any degree of degeneration. The arrangement of the cells within this group was undoubtedly spherical. Those in the outer regions were significantly larger than those inwardly situated. There was no evidence of a suspensor having been formed.

A similar arrangement was encountered in a group of embryonic cells (cf. Fig. 5) located in a chamber but, although both masses were similar in general appearance, there were significant differences. There was no enclosing jacket seen in the second instance, nor was there any evidence of jacket-cell remains. The nuclei were smaller than those of the first mass and there was a decrease in the amount of cytoplasm. The first group described was found in tissue excised on July 5, 1954, and the second in tissue excised on July 11, 1954.

Shown in Figure 11 is a mass of substance of very unusual appearance, located in the chamber. Such has never been recorded as occurring in this material *in vitro*. It appears to consist of an irregular mass of cytoplasm with nuclei and nuclear material dispersed throughout. The nuclear material occurred as irregular masses or as groups of structures which resembled chromosomes. Two other instances of this unexpected phenomenon were noted in the material studied but they were not so large or distinct.

Discussion

The results regarding the growth of the proembryos *in vitro* in the various media indicate that yeast extract (or an equivalent of this) is necessary in the medium for successful culturing. The results are not conclusive enough to warrant the selection of dextrose or sucrose as the most suitable carbohydrate for the basic medium, but it is thought that sucrose is probably the better sugar. This is substantiated in the literature (8, 13, 14).

As penicillin was ineffective in the concentration used, the low incidence of contamination is attributed to the precautions taken during the dissection.

The irregularity in the time of fertilization observed in this work and reported by Woods (15) suggests that the development of the ovule determines the time of fertilization and that its development is in turn controlled by the environment.

To discuss the alterations in the growth of the proembryo of *Pinus* caused by the environment *in vitro*, it would be advisable to review briefly the condition *in vivo*. The following account is derived from the literature (2, 5) and from observation of the *in vivo* material.

Upon fertilization, two diploid nuclei are formed and undergo mitosis, forming four nuclei, which become aligned in a plane perpendicular to the axis of the archegonium and at the basal extremity of its axis. There, partial cell walls are formed such that one end remains exposed to the egg cytoplasm. Mitosis follows to produce two tiers of four cells each; those of the lower tier have complete cell walls and those of the upper tier have incomplete ones. Two separate series of mitoses occur to produce three and then four tiers of four cells each. All those except the ones of the upper tier have complete cell walls.

The cells of the second tier (from the bottom) begin to elongate and become suspensorial. This forces the cells of the bottom tier into the channel created in the prothallium. The cells of the upper two tiers remain in the chamber; those of the third tier are the rosette cells which can, under special conditions, produce proembryos. The continued elongation of the suspensors forces the tip cells further into the channel, where it is thought they enter into competition for the available nutriments. There can be a total of twenty-four proembryos in the same channel if all six possible archegonia are fertilized. However, some of these are never formed and others never grow beyond a few cells. Approximately two weeks after fertilization only three or four proembryos can be seen, and these are usually quite large.

Although there are several embryonic cells or groups of cells present in the chamber and channel in the early stages of embryonic development, in normal circumstances only one proembryo develops into the immature plant of the seed. Thus many proembryos abort. This may be due to lack of nutrient or it may be an effect of genetic control. However, placement of the excised units in artificial medium seems to prolong the lives of the ill-fated masses and so more embryonic masses are seen *in vitro* than *in vivo*, and a phylogenetic factor is not apparent.

There are several possible sources for these groups of cells. The cells of the upper tier, which are apparently non-functional, could be capable of reproducing under *in vitro* conditions. Also the rosette cells which have been known to produce proembryos *in vivo*, should show an increase in this tendency when placed in culture. These two groups of cells would be responsible for the many embryonic groups seen in the chamber, especially the small or medium-sized ones, as the source of the small groups of embryonic cells was probably unicellular. Whatever the source, it would seem that the artificial nutriment has stimulated growth at different points and at an abnormally large number of centers.

Another potential source would be those proembryos which under *in vivo* conditions were apparently losing in the competition for survival. These would account for the masses seen in the channel, especially those located near the mouth of the chamber. The size of the resulting mass would depend on the size of the initial group. It was found *in vitro* that the large masses were most frequently located well down in the channel, and their source was the proembryos that were still vigorous at the time of trans-

plantation. Those masses associated with the edges of the channel were probably derived from the remnant of large masses which had declined *in vivo* before inoculation. Thus these groups, seen *in vitro*, were the small- or medium-sized groups, since their initial source had probably been reduced to a few cells.

Media containing yeast extract as well as sugars, were generally found to be the best for reviving and initiating proembryonic growth. This is substantiated by the fact that more groups were seen in individual pieces of tissue grown in such media than in those grown in other media. Also, these groups were larger in this type of medium than those in the others.

The degree of radiality expressed by a group of embryonic cells was determined by its size at the time of inoculation, by its position in the channel or chamber, and by the medium in which it was cultured. The larger groups, by virtue of their more advanced although still slight differentiation, were less able to undergo three-dimensional growth. Small- or medium-sized groups, which probably originated from single or quite small groups of cells, were relatively undifferentiated at the time of their inoculation, and so were free to grow in three dimensions. Also, the presence of physical barriers, such as the edges of the channel or other prothallial remnants, would hamper this tendency. The lower-situated groups tended to have a low degree of radiality owing both to the presence of the physical barrier of the channel edges and to their relatively advanced differentiation. Small groups located in the chamber or high in the channel had neither of these apparent limitations. Possibly because of this, they indicated a high degree of radiality. Groups associated with the edges of the channel generally showed a low degree of radiality, in spite of their small size. This could be attributed to their position next to the channel edge and to the presence of cellular remains observed about the masses.

When the medium is one which supports abundant growth, these previously mentioned factors were no longer critical for the expression of radiality. This is corroborated by the appearance, in the lower portion of the channel, of large masses possessing a high degree of radiality.

The large masses found in the upper regions of the channel and in the chamber, could have been placed there by the mechanical treatment of the tissues in the course of the processing. Proembryos are not fixed in their positions in the channel, but rather, they are suspended in it by their suspensors. Thus, such loose masses could be shaken free and relocated in another position. If this had occurred before or during inoculation, these groups would have had a greater opportunity for radial growth, as the physical barrier of the channel edges would have been removed. Those groups in the chamber that showed a degree of radiality comparable to that of the masses located in the lower regions of the channel were probably relocated after their removal from the media.

This postulated relocation of embryonic groups would account for those masses whose growth directions were observed at various angles to that of

the normal direction or completely reversed with respect to the normal. However, since growth was observed to occur in these abnormally oriented masses, it would seem that the direction of growth was determined by their differentiation and not by their orientation. This is significant as it would indicate that the direction of growth *in vivo*, which is parallel to the axis of the archegonia and directed away from it, is determined not by genetic factors alone, but by the chemical and physical factors of the environment within the prothallium.

The foregoing results and discussion indicate that physical and chemical factors do affect the expression of growth in embryonic cells and so this growth is not controlled entirely by genetic factors. This is contrary to the "primitive spindle" theory, advanced by Bower (1) which endeavors to establish the premise that filamentous organization underlies all embryonic development in plants, even the more advanced forms. Both Bower and Lang (7) hold that this filamentous organization, especially in the formation of the suspensor, has phylogenetic significance, *harking back to a filamentous algal organization.*

As one of the authors (10) indicated, this would lead to the view that the filamentous type of suspensor is more primitive than the conical type and this violates the natural phylogenetic sequence. He has shown that the onset of the suspensor as well as asymmetrical growth in *Ginkgo* can be deferred *in vitro*. Thus, he regards the suspensor, under natural conditions, as a secondary feature imposed upon the symmetrical growth of the early stages of proembryonic development by external conditions, in this case the surrounding tissue. Woods (15) has substantiated this in his work with *Pinus*. He found that the development of the suspensor can be impeded by *in vitro* treatment and he concluded that there is little, if any, phylogenetic significance in the presence or absence of a suspensor, its abolition being a physiological response.

This view is conclusively substantiated in the present work as illustrated in the material shown in Figure 6. This is a group of embryonic cells whose origin was probably the first nuclei formed after fertilization. Since this material was excised within the recorded period of fertilization it is probable that fertilization had occurred just previous to, or shortly after, inoculation. The nuclei resulting from fertilization probably did not align themselves in the customary plane, for if they had, the expression of growth would not have been so definitely spherical. The presence of the intact, undistorted jacket gives further evidence that the origin of this group of cells was the nuclei resulting from fertilization. The complete absence of any indication of suspensor-development indicates that this feature is determined in its occurrence by physical and chemical factors, although genetic factors may give the material the potentialities for its development.

The occurrence of the second group (cf. Fig. 5), similar in appearance, substantiates the statement that the expression of growth, in an environment uniform with respect to chemical and physical factors, is spherical when the

origin of that growth is a single or small group of cells. The origin of the second group of cells could not have been the same as that of the first group as there was a distinct difference in the condition of the cells and in the size of the nuclei of the two groups. Also there was no indication of the presence of a jacket. The fact that this group was excised 6 days after the end of the fertilization period was the deciding factor in considering that the origin of this group was probably not those cells formed soon after fertilization. It could have originated from the rosette cells.

The mass of material in Figure 11 seems to suggest that nuclei can lose all normal features and become a disorganized (not disintegrating) mass of material. Somewhat similar though less disorganized masses were noted by Straus (11) in maize endosperm grown *in vitro*. Straus referred to a disorganization which occurs after treatment with colchicine, which was similar to abnormal mitosis in untreated human cancer cells. The reason for the occurrence in this material is not apparent. It is, however, very important in assessing the effects of growth-controlling agents which, in future, will be added to the basic medium, the nature of which has been suggested in this work.

Summary and Conclusions

In this work, proembryos of *Pinus* were found to be suitable material for the *in vitro* study of factors influencing the expression of growth. From the media used, useful information was obtained regarding the requirements of the basic medium.

Although the evaluation of the effects of an alteration in the environment of growing cells is only relative, the results clearly indicate that chemical and physical factors influence the expression of inherited potentialities. The development of the proembryos of *Pinus* is not controlled entirely by phylogenetic factors, and shows *in vitro* the adoption of radial or three-dimensional growth rather than a filamentous organization.

These results substantiate conclusively the hypothesis set forth by Radforth (10) regarding the nature of suspensorial formation.

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